Curcumin potentiates the antitumor effects of 5-FU in treatment of esophageal squamous carcinoma cells through downregulating the activation of NF-κB signaling pathway in vitro and in vivo

Fang Tian1††, Tianli Fan2†, Yan Zhang3, Yanan Jiang1, and Xiaoyan Zhang1

1Department of Pathophysiology, School of Basic Medicine, Zhengzhou University, Zhengzhou 450001, China
2Department of Pharmacology, School of Basic Medicine, Zhengzhou University, Zhengzhou 450001, China
3Department of Anesthesia, the First Affiliated Hospital, Zhengzhou University, Zhengzhou 450001, China
†These authors contributed equally to this work.
*Correspondence address. Tel: +86-371-67739263; Fax: +86-371-68762299; E-mail: tianfang715@yahoo.com.cn

Although constitutive activation of nuclear factor-kappaB (NF-κB) signaling pathway has been reported in multiple different human tumors, the role of NF-κB pathway in esophageal squamous cell carcinoma (ESCC) remains ill-defined. Abundant sources have provided interesting insights into the multiple mechanisms by which curcumin may mediate chemotherapy and chemopreventive effects on cancer. In this study, we first analyzed the status of NF-κB pathway in the two ESCC cell lines Eca109 and EC9706, and then further investigated whether curcumin alone or in combination with 5-fluorouracil (5-FU) could modulate NF-κB pathway in vitro and in vivo. The results showed that NF-κB signaling pathway was constitutively activated in the ESCC cell lines. Curcumin suppressed the activation of NF-κB via the inhibition of IκBα phosphorylation, and downregulated the expressions of Bcl-2 and CyclinD1 in ESCC cell lines. Curcumin combined with 5-FU led to the lower cell viability and higher apoptosis than 5-FU treated alone. In a human ESCC xenograft model, curcumin or 5-FU alone reduced the tumor volume, but their combination had the strongest anticancer effects. Besides, curcumin could also inhibit NF-κB signaling pathway through downregulation of the IκBα phosphorylation and induction of cell apoptosis in vivo. Overall, our results indicated that constitutively activated NF-κB signaling pathway exists in the two ESCC cells and the chemopreventive effects of curcumin were associated with downregulation of NF-κB signaling pathway and its downstream genes.

Keywords esophageal squamous cell carcinoma; curcumin; NF-κB signaling pathway; chemoprevention; nude mice

Received: June 6, 2012 Accepted: July 20, 2012

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in developing countries, especially in northern China [1]. Although the therapy strategies have been improved, the prognosis of patients with ESCC is still poor. Moreover, cells of ESCC are known to develop resistance to chemotherapeutic drugs, thus, resulting in a dramatic decrease in the 5-year survival rate of ESCC patients. 5-Fluorouracil (5-FU) is frequently used in combination chemotherapy of ESCC, but some patients have a poor response to 5-FU-based chemotherapy. In addition, numerous toxic effects occur at doses required for efficacy, including myelosuppression, fever, nausea, and vomiting. Thus, understanding the molecular mechanisms by which ESCC develops will allow us to design new therapeutic strategies to improve the clinical outcome and tolerance for this disease.

A major idea today is that cancer may be prevented or treated by targeting the products of specific cancer-related genes, frequently encoding signaling proteins or transcription factors. Recently, a large amount of evidence has demonstrated that nuclear factor-kappaB (NF-κB/Rel) plays an essential role in carcinogenesis. A variety of extracellular stimulus factors such as inflammatory cytokines, growth factors, DNA damaging agents, bacterial and viral products trigger a common signal transduction pathway based on the phosphorylation, ubiquitination, and proteosome-dependent degradation of IκB to freely activated NF-κB, which is then rapidly translocated into the nucleus and binds to the promoter region of the relevant downstream genes to evoke a series of transcriptional events [2]. Thus, the phosphorylation of IκBα is an indispensable step to activate NF-κB signaling pathway, which is catalyzed by an IκB kinase (IKK) [3].
Constitutive activation of NF-κB signaling pathway has been investigated in many cancers including hepatocellular, colonic, pancreatic, and cervical cancers [4–7]. Thus, the elucidation of molecular and cellular targets critical in cancer development and prevention is an area of intensive research and is driving the development of highly specific small-molecule inhibitors, which may either prevent carcinogenesis, curtail its progression, or even cure the disease [8]. Curcumin, an extract from the root of Curcuma longa L, as an anti-oxidation and anti-inflammation component, has been widely used in Indian medicine. More recently, curcumin has been used in cancer therapy alone or as a chemotherapy adjuvant. For example, some studies have revealed that curcumin inhibits cell proliferation and induces apoptosis in human leukemia, prostate cancer, and non-small-cell lung cancer cell lines [9–11]. Numerous clinical trials have indicated that curcumin is quite safe when administered even at a daily dose of 12 g for 3 months [12]. In our previous study, elevated expression of NF-κB signaling pathway has been reported in ESCC cell lines [13,14]. To confirm whether curcumin alone or in combination with 5-FU has the effect of chemoprevention on ESCC, in this study we used two ESCC cells and a nude mouse xenograft model and further explored the mechanism mediated by curcumin. The results suggested that constitutive NF-κB activation played an important role in the survival of ESCC cells, and curcumin downregulated the phosphorylation of IkBα, thereby decreasing the expression of anti-apoptotic protein Bcl-2 and arresting cell cycle by preventing the expression of cyclin D1. Therefore, curcumin may be a useful agent for the chemoprevention of ESCC.

Materials and Methods

Antibodies
Mouse monoclonal antibodies to p65 (sc-8008), p-IκBα (sc-8404), IKKβ (sc-8014), and Bcl-2 (sc-7382) rabbit polyclonal antibodies to IkBα (sc-371), p50 (sc-114), and cyclin D1 (sc-753) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

Cell lines and cell culture
Two human ESCC cell lines, Eca109 and EC9706, were provided by State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Science (Beijing, China). Cervical cancer cell line HeLa229 was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China) as the positive control. All cell lines were cultured in RPMI1640 medium (GIBCO-BRL, Carlsbad, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂. Curcumin (Sigma, St Louis, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma) to make the stock concentration at 100 μM.

Preparation of cytoplasm and nuclear proteins
Cytoplasm and nuclear proteins were respectively extracted using nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, USA) from the three cells until 90% confluence. Aliquots of the proteins were stored at −70°C and the protein concentrations were determined by Bradford protein-binding assay.

Western blot analysis
Cytoplasm or nuclear protein (50 μg) from each cell line was separated on sodium dodecyl sulfated-polyacrylamide electrophoresis gels (SDS-PAGE), along with 20 μl of pre-stained protein molecular weight marker (Fermentas, Glen Burnie, USA) as a standard. The proteins were electro-transferred to nitrocellulose membranes (Hybond-c pure, Amersham, Piscataway, NJ) in transfer buffer containing 25 mM Tris, 193 mM glycine, and 20% methanol. The membranes were incubated with 5% skimmed milk in Tris-buffered saline plus 0.05% Tween 20 (TBST) at room temperature (RT) for 2 h, and then rinsed three times in TBST and incubated with anti-p50, anti-p65, anti-IκBα, anti-P-IκBα, anti-IκKβ antibodies, anti-p-IκBα, CyclinD1, and Bcl-2 antibodies diluted in 1% skimmed milk (1 : 100), respectively, at RT for 2 h. The blots were rinsed three times in TBST and incubated with 1 : 5000 dilution of goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at RT. After extensive washing with TBST, proteins were visualized on the membrane developed with DAB according to the manufacturer’s instructions.

Electrophoretic mobility-shift assay
The cells were treated with curcumin (50 μM) for various times (from 0 to 60 min), and the nuclear proteins extracted from cells were analyzed by electrophoretic mobility-shift assay (EMSA). In brief, consensus NF-κB DNA-binding oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ was labeled with Biotin 3′ end labeling kit (Pierce). The nuclear proteins (10 μg) were incubated for 20 min at RT with biotin-labeled DNA probes in 20 μl of reaction mixture containing 10 M Tris-HCl, pH 8.5, 0.5 mM ethylene-diaminetetraacetic acid (EDTA), 5 mM MgCl₂, 1 M KCl, 0.05% NP40, 2.5% glycerol, 1 μg/μl poly (dl-dC), and 0.2 μg/μl bovine serum albumin. Nucleo-protein complexes were loaded onto the pre-electrophoresis 6% non-denaturing polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at 100 V for 2 h at RT. The electrophoresed binding reactions were transferred to nylon membrane (Hybond-N⁺, Amersham) via capillary transfer system at RT overnight. The biotin end-labeled DNA probe was detected using the

streptavidin-HRP conjugate and chemiluminescent substrate. The membranes were exposed to X-ray film for 2–5 min to obtain the perfect signal.

Cell proliferation assay
To test the proliferation effect after curcumin exposure alone or in combination with 5-FU, EC9706 and Eca109 cells were seeded at a concentration of $3 \times 10^3$ in 96-well plates and incubated overnight. Curcumin (50 μM) alone or in combination with 5-FU (327 μg/ml) were added into the cultured cells for 24, 48, and 72 h. After rinsing with phosphate-buffered saline (PBS), 20 μl MTT (Sigma) (final concentration 5 mg/ml) was added to the medium. Cells were incubated at 37°C for 4 h. The medium was removed and the precipitated formazan was dissolved by adding 200 μl DMSO, shaking for 20–30 min. The absorbance was detected using a reader at 560 nm.

Annexin V—propidium iodide staining for apoptotic cells
Cells were seeded in 6-well plates and incubated overnight. Next, cells were treated with 50 μM curcumin alone or in combination with 5-FU (327 μg/ml) for 72 h. These cells were harvested by rapid trypsinization, washed twice with PBS, and then adjusted at $1 \times 10^5$. The certain number cells were stained with fluorescein isothiocyanate (FITC)—annexin V—propidium iodide (PI) with annexin V—FITC Kit (Beckman Coulter, Brea, USA) according to the protocol. Stained cells were placed on ice and protected from the light for 10 min until they were evaluated on flow cytometry (BD FACS Calibur and Cell Quest 3.0, Franklin Lakes, USA). Viable cells are those that stain negatively for both annexin V and PI. Cells in early apoptosis are positive for annexin V and negative for PI, whereas those that are necrotic or in late apoptosis are positive for both annexin V and PI.

Animals
Male athymic BALB/c nude mice were purchased from the Laboratory Animal Ltd in Shanghai, China. The animals at 4–5 weeks of age were housed five per cage in an air-conditioned room at temperature of 25–26°C with the humidity of ~50%, lit 12 h/day. All animal studies were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of Henan Province, China.

Animal treatments
EC9706 cells were harvested from 70% to 80% confluent cultures by exposure to trypsinase and then washed, resuspended in PBS at $2 \times 10^7$ cells/ml. Only single-cell suspensions with 90% viability were used for injection. A cell suspension of 200 μl ($4 \times 10^6$ cells) was inoculated subcutaneously (s.c.) into the right flank of athymic mice [15]. After 1 week of implantation, the tumors were measured in three dimensions and the tumor volume was calculated according to the formula $V = \frac{1}{2}ab^2$, where $a$ and $b$ represent the length and the width of tumor measured with sliding caliper, respectively. The animals were monitored for tumor growth every other day.

When tumor size reached 100–200 mm$^3$, tumor-bearing animals were randomly assigned to the following four groups ($n = 5$): (i) untreated control (PBS); (ii) curcumin (50 μM/one animal) alone [16]; (iii) 5-FU (10 mg/kg) alone; and (iv) curcumin (50 μM/one animal) + 5-FU (10 mg/kg). Curcumin or 5-FU was intraperitoneally injected every 3 days for 21 days. In the curcumin combined with the 5-FU group, animals were treated with curcumin and 5-FU on alternative days. After 3 weeks of treatment, the mice were killed. Tumor volumes were compared among groups using unpaired Student’s $t$-test. Inhibition rate = [(tumor weight of control group − tumor weight of experimental group)/tumor weight of control group] × 100%. Tumor tissue was immediately fixed in 4% buffered paraformaldehyde overnight for immunohistochemistry and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) analysis.

Immunohistochemical analysis
Tissue sections of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene followed by the treatment with a graded series of alcohol and distilled water, and washed thoroughly with PBS. Antigen retrieval for paraffin-embedded tissues was performed with sodium citrate 0.01 M (pH 6.0) and then the container was placed in boiled water for 20 min. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in methanol for 10 min. The sections were washed three times with PBS and incubated for 30 min at RT with a protein blocking solution (containing 10% normal rabbit serum in PBS). After that, the samples were incubated with primary antibodies: mouse monoclonal antibodies to p-IκBα and rabbit polyclonal antibodies to IκBα (1 : 200; Santa Cruz), as well as PBS (negative control), respectively, at 4°C overnight. The samples were then rinsed three times with PBS and subsequently incubated for 30 min with the appropriate dilution of the secondary antibody, followed by incubation with the HRP-linked streptavidin biotin complex for 10 min at RT. Positive reactions were visualized by incubating the slides with DAB for 5 min. The sections were then washed three times with PBS, counterstained with hematoxylin for 15 s, dehydrated and cleared and mounted. Positive cells from five fields was examined and counted from each group.

TUNEL assay
In brief, the tissue sections were deparaffinized in xylene, treated with a graded series of alcohol and distilled water,
and washed thoroughly with PBS. The slides were incubated with proteinase K (20 μg/ml in PBS) for 20 min at RT, and TUNEL staining was carried out using the in situ cell death detection kit (KeyGen Biotech Ltd., Nanjing, China) according to the manufacturer’s instructions. TUNEL-positive cells from five independent fields were counted manually.

Statistical analysis
The results were expressed as mean ± standard deviation, except as otherwise stated. The results were analyzed by standard χ² test and one-way analysis of variance, respectively, using SPSS version 13.0 (SPSS, Chicago, USA). P < 0.05 was considered statistically significant.

Results

Activation of NF-κB signaling pathway in ESCC cell lines
Western blot analysis showed that the levels of p50, p65, IkBα, p-IκBα, and IKKβ in the cytoplasm extracts of the Eca109 and EC9706 cell lines were similar compared with those in the control cell line (HeLa229) [Fig. 1(A)]. The expression levels of p50 and p65 in the nuclear extracts of the ESCC cell lines were higher compared with those in HeLa229 [Fig. 1(B)], which indicated that p50 and p65 could translocate to the nucleus to regulate the downstream genes.

Curcumin inhibited the activation of NF-κB signaling pathway and downregulated the expression of NF-κB-regulated gene in ESCC cell lines
Western blot results indicated that curcumin inhibited IkBα phosphorylation in the two ESCC cell lines after treatment with 50 μM curcumin at the different time point (0–60 min) [Fig. 2(A)], which indicated that curcumin inhibited IkBα phosphorylation in a time-dependent manner. EMSA results showed that curcumin suppressed the NF-κB-DNA-binding in a time-dependent manner [Fig. 2(B)]. In this study, we detected the effects of curcumin on the expression of Bcl-2 and cyclin D1 in two ESCC cells. Figure 2(C) showed that curcumin downregulated the expression of Bcl-2 and cyclin D1 in a time-dependent manner.

Curcumin alone or combined with 5-FU inhibited the proliferation of ESCC cells in vitro
To investigate whether inhibition of the activity of NF-κB by curcumin could increase the sensitivity of 5-FU, we compared the effect of curcumin alone or combined with 5-FU on proliferation of ESCC cell lines. The results showed that curcumin inhibited cell proliferation and increased the sensitivity to 5-FU (Fig. 3).

Curcumin alone or combined with 5-FU induced the apoptosis of ESCC cells
The results showed that when the cells were incubated with curcumin alone (50 μM), the percentage of apoptotic cells in EC9706 and Eca109 was higher than that in the control group. When the cells were treated with curcumin plus 5-FU (327 μg/ml), the percentage of apoptotic cells and death cells in EC9706 and Eca109 was significantly increased, which indicates that curcumin could enhance the sensitivity of the ESCC cells to 5-FU (Table 1, P < 0.05).

Curcumin enhanced the antitumor effects of 5-FU in ESCC xenografts model
Owing to the abnormally high activity of the NF-κB signaling pathway in ESCC cell lines, the effect of curcumin alone or in combination with 5-FU on the growth of ESCC xenografts was evaluated in the transplantable tumor of EC9706. The therapy was initiated on the Day 7 after tumor implantation when there was no difference in the volume of tumor among groups (P > 0.05). These tumor-bearing mice were randomly

Figure 1 NF-κB signaling pathway is constitutively activated in two ESCC cell lines
(A) Cytoplasm protein extracts (50 μg/lane) from the three cell lines were detected with anti-p50, p65, IkBα, p-IkBα, and IKKβ antibodies. Proteins were visualized with DAB staining. Equal protein loading was controlled with anti-actin antibody. (B) Nuclear protein extracts (50 μg/lane) from the three cell lines were detected with anti-p50 and anti-p65 antibodies. Proteins were visualized with DAB staining.
Curcumin potentiates the antitumor effects of 5-FU on ESCC in vitro and in vivo

Figure 2 Curcumin inhibits IκBα phosphorylation and downregulates the expression of NF-κB-regulated gene products  
(A) ESCC cells were treated with curcumin (50 μM) for different times, and cytoplasm protein extracts (50 μg/lane) were subjected to electrophoresis on 12% SDS-PAGE and were performed with anti-p-IκBα antibody. Proteins were visualized with DAB staining. Equal protein loading was controlled with anti-actin antibody. (B) ESCC cells were treated with curcumin (50 μM) for different times. Nuclear extracts were prepared and analyzed by EMSA using an oligonucleotide which contains the consensus binding site for NF-κB. Data are representative of three separate experiments. (C) ESCC cells were treated with curcumin (50 μM) for indicated times and then the cytoplasmic extracts were prepared and resolved on 12% SDS-PAGE. Proteins were visualized with DAB staining. Equal protein loading was controlled with anti-actin antibody.

Figure 3 Curcumin alone or combined with 5-FU inhibits the proliferation of two ESCC cells  
Cells were treated with curcumin alone (50 μM) or combined with 5-FU (327 μg/ml) at indicated time. Cells viability was analyzed by MTT assay. Data are mean values ± standard errors from triplicate experiments. *P < 0.05 compared with control.

Table 1 Apoptosis rate of EC9706 and Eca109 detected by flow cytometry

<table>
<thead>
<tr>
<th>Group</th>
<th>EC9706 (%)</th>
<th>Eca109 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.03 ± 0.08</td>
<td>7.66 ± 0.25</td>
</tr>
<tr>
<td>Curcumin</td>
<td>17.80 ± 0.59*</td>
<td>26.00 ± 0.10*</td>
</tr>
<tr>
<td>5-FU</td>
<td>20.54 ± 0.71</td>
<td>28.20 ± 0.37</td>
</tr>
<tr>
<td>Curcumin + 5-FU</td>
<td>38.01 ± 0.33**</td>
<td>43.45 ± 0.50**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with control group; **P < 0.05, compared with 5-FU group.

in curcumin or 5-FU alone group was significantly decreased as compared with that in the control group (P < 0.05) (Table 2) while curcumin combined with 5-FU significantly inhibited the growth of tumor compared with 5-FU alone (P < 0.05) (Fig. 4). All animals were killed on day 28, and the tumor weight was measured. The inhibition rates of curcumin, 5-FU, and curcumin + 5-FU groups were 34.78%, 45.22%, and 83.48%, respectively, compared with the control (Table 2).
Table 2 Effects of curcumin alone or combined with 5-FU on the growth of human ESCC xenografts in nude mice (n = 5)

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal weight</th>
<th>Tumor volumea before therapy</th>
<th>Tumor volumea after therapy</th>
<th>Inhibition of rate of tumor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.13 ± 1.46</td>
<td>147.09 ± 40.77</td>
<td>1703.25 ± 491.18</td>
<td>0</td>
</tr>
<tr>
<td>Curcumin</td>
<td>20.25 ± 1.68</td>
<td>154.66 ± 47.23</td>
<td>1306.25 ± 228.41</td>
<td>34.78</td>
</tr>
<tr>
<td>5-FU</td>
<td>20.70 ± 1.17</td>
<td>152.38 ± 45.36</td>
<td>986.50 ± 210.59</td>
<td>45.22</td>
</tr>
<tr>
<td>Curcumin + 5-FU</td>
<td>21.22 ± 1.44</td>
<td>149.56 ± 40.12</td>
<td>604.75 ± 159.59*</td>
<td>83.48*</td>
</tr>
</tbody>
</table>

*Tumor volume in mm³.

*P < 0.05, compared with control group.

Curcumin inhibited NF-κB signaling pathway through the downregulation of the pIkBα

To explore whether the antitumor effect of curcumin in vivo was also achieved via inhibition of NF-κB signaling pathway, the phosphorylation status of IkBα in the xenografts were detected by immunohistochemistry. pIkBα was mainly expressed in the cell cytoplasm (Fig. 5) and the number of positive cells expression pIkBα were 324, 97, and 81 cells/1500 cells in control, curcumin, and curcumin + 5-FU groups, respectively. There was a significant difference when the curcumin or curcumin + 5-FU groups were compared with the control group (P < 0.05).

Curcumin induces cell apoptosis in the tumor tissues

The antitumor effects of curcumin on ESCC xenografts broached the question that what is the mechanism of the biological events caused by curcumin in vivo. As shown in Fig. 6, TUNEL staining showed that there were 56, 40, 87, and 4 apoptosis cells/1500 cells in curcumin, 5-FU, curcumin + 5-FU, and control groups, respectively, and there was a significant difference between each treated group and control group (P < 0.05). Obviously, the apoptosis cells in curcumin + 5-FU group were higher than those in curcumin or 5-FU alone group (P < 0.05), indicating that curcumin is highly effective in potentiating the apoptosis effects of 5-FU.

Discussion

Some reports have revealed that NF-κB is an important regulator for several genes involved in cell survival, transformation, differentiation, invasion, and growth of the cancer cells [17–19]. Chemoresistance has also been linked with the activation of NF-κB signaling pathway [20]. 5-FU has been extensively used as the front-line chemotherapeutic agent in esophageal cancers. In a study on salivary gland cancer cells, 5-FU-induced apoptosis in cancer cells is due to the suppression of NF-κB activity [21]. Tamatani et al. [22] also showed that inhibition of NF-κB signaling pathway in human oral cancer, as a rational approach, would improve conventional radiotherapy and chemotherapy outcomes. Some evidence suggests that certain dietary components might be used in combination with traditional chemotherapeutic agents to treat cancer [23]. The effects of curcumin in mantle cell lymphoma (MCL) cells are in agreement with the previous report that curcumin inhibits the constitutive NF-κB and IKK leading to the inhibition of expression of NF-κB-regulated gene products, which results...
in the suppression of proliferation, cell cycle arrest, and induction of apoptosis in MCL [24]. Thus, curcumin may be a potential candidate in the control of various cancers [25–27].

Chemoprevention is a relatively novel and promising approach for controlling cancer, which uses specific natural products or synthetic agents to suppress, reverse, or prevent premalignancy before transformation into invasive cancer. Curcumin derived from the root of the plant *C. longa* is a yellow pigment commonly used as a coloring agent in foods. Recent studies have shown that curcumin downregulates NF-κB signaling pathway and may be a useful agent in the treatment of cancers [10,28–30].

Our study showed that the signaling pathway of NF-κB was activated in the two ESCC cell lines. Furthermore, we found that curcumin inhibited IκBα phosphorylation in a

---

**Figure 5** Expression of pIκBα protein in tissue of EC9706 xenograft treated with different methods  
(A) The representative pictures of pIκBα staining with different treatments. (× 400). (B) The quantification of positive cells of pIκBα with different treatments. *P < 0.05, compared with control group.

---

**Figure 6** Curcumin in combination with 5-FU triggers cell apoptosis in tumor tissue  
TUNEL staining showed that curcumin enhanced the effect of 5-FU by inducing apoptosis in ESCC xenografts. (A) negative control. (B) curcumin group. (C) 5-FU group. (D) curcumin + 5-FU group. The apoptotic cells were counted from 5 animals in each group (× 400).
time dependence and the suppression of NF-κB activity by curcumin was accompanied by obvious anti-proliferative activity in the two ESCC cell lines. Activation of NF-κB signaling pathway leads to expression changes of the genes associated with apoptosis and cell-cycle regulation, such as Bcl-2, cyclin D1, COX-2, and MMP-9. In this study, Bcl-2 and cyclin D1 were detected in two ESCC cells, and their expression was downregulated by curcumin through inhibition of NF-κB signaling pathway. The results also showed that curcumin alone inhibited the cell proliferation and induced apoptosis in two ESCC cells. In addition, when curcumin was combined with 5-FU, cell viability remained lower than those treated with the single agents. Anti-apoptosis of tumors is a main disadvantage in chemotherapy of cancers clinically. Combination chemoprevention pursues the ability to enhance the chemopreventive efficacy of both agents (synergism), while reducing side effects by dose reduction. Therefore, the apoptosis-induced ability of curcumin, in combination with 5-FU, can make it a potentially effective anti-cancer assistant agent and sensitize tumor cells to chemotherapy drugs. Thus, in vitro results using various methods showed curcumin and 5-FU alone are quite efficacious in inhibiting the proliferation and inducing apoptosis, while the two agents together are much more effective. The possible mechanism is that the activation of NF-κB signaling pathway plays an important role in the esophageal tumorigenesis, while curcumin downregulates the NF-κB activation and its regulated gene products.

To investigate the inhibitory effect of curcumin on growth of the ESCC cells in vivo, EC9706 cells were inoculated s.c. into nude mice. The results showed that both curcumin and 5-FU could induce obvious tumor inhibition, especially when the tumor was treated with the combination of curcumin and 5-FU. As compared with control, curcumin or 5-FU alone resulted in the inhibition of tumor growth. However, the combination of curcumin and 5-FU induced a very robust antitumor activity compared with the use of each treatment individually (P < 0.05). In addition, curcumin could inhibit NF-κB signaling pathway through downregulation of the IκBα phosphorylation and degradation of IκBα. The overall apoptosis in the tumor tissue, as indicated by the TUNEL staining, was significantly increased by curcumin combined with 5-FU. Therefore, the inhibition effect of curcumin on the growth of ESCC in vivo might be through blocking NF-κB signaling pathway and inducing cell apoptosis.

Our previous study has showed that RNAi targeting for p65 increases the sensitivity of the ESCC cells to 5-FU [13]. However, the method of RNAi has a significant shortcoming with short acting time, which greatly limits its clinical use. Since curcumin has minimal systemic side effects, it is likely an ideal candidate for clinical use, alone or combined with other agents [31]. Curcumin, which has been found to be highly safe and well tolerated even at very high dose [12], is in clinical trial for the treatment of pancreatic cancer [32].

Data from both in vitro and in vivo cancer models indicated that constitutively activated NF-κB signaling pathway may be an important mechanism responsible for survival and proliferation of the EC9706 cells. Curcumin alone or in combination with 5-FU can be used as an adjuvant agent through enhancing the sensitivity of antitumor agents in treatment of this cancer. Therefore, suppression of NF-κB signaling pathway may be a potential target for the chemoprevention strategies in ESCC.

**Funding**

This study was supported by Grants from the National Natural Science Foundation of China (81071723), Research Fund for the Doctoral Program of Higher Education of China (20104101120002), University Key Teacher by the Henan Educational Committee (2011GGJS-010), and Zhengzhou Medical Science and Technique Foundation (0910SGYS3389-7).

**References**

Curcumin potentiates the antitumor effects of 5-FU on ESCC in vitro and in vivo


