

Curcumin inhibits the invasion of lung cancer cells by modulating the PKC α /Nox-2/ROS/ATF-2/MMP-9 signaling pathway

ZHIGANG FAN^{1,2}, XIAOYI DUAN³, HUI CAI^{1,4}, LI WANG^{1,5}, MIN LI¹, JINGKUN QU¹,
WANJUN LI^{1,6}, YONGHENG WANG^{1,7} and JIANSHEG WANG¹

¹The Second Department of Thoracic Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an;

²Department of Oncology, No. 3201 Hospital of Xi'an Jiaotong University, Hanzhong; Departments of ³Nuclear Medicine and ⁴Anesthesia, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an; ⁵Department of Internal Medicine, Hospital of Chang'an University, Xi'an; ⁶Department of Pathology, No. 3201 Hospital of Xi'an Jiaotong University, Hanzhong; ⁷Provincial Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, P.R. China

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Abstract. Invasion and metastasis are the major causes of tumor-related mortality in lung cancer. It is believed that curcumin is an effective drug possessing anti-invasive and anti-metastatic activities in the treatment of cancer. However, the specific mechanisms remain unclear. In the present study, we investigated whether the PKC α /Nox-2/ATF-2/MMP-9 signaling pathway is involved in the invasive behavior of lung cancer and whether curcumin could inhibit invasion by modulating this pathway. The cytotoxic effect of curcumin was evaluated by MTT assay and the capacity of invasion was assessed by Transwell assay. siRNA and plasmid transfection techniques were used to study the function of targeted genes. Real-time PCR and western blot analysis were used to evaluate the expression levels of PKC α , Nox-2, MMP-9 and the phosphorylation of ATF-2. The results showed that curcumin inhibited the proliferation and invasion of A549 cells in a dose-dependent manner. Overexpression of MMP-9 enhanced the invasion of A549 cells. However, inhibition of MMP-9 by siRNA or curcumin suppressed cell invasion. Moreover, we also demonstrated the catalytic role of PKC α in expression of MMP-9 and cellular invasion in A549 cells, which was dependent on the expression of Nox-2 and phosphorylation of ATF-2. Finally, we also showed that curcumin dose-dependently reduced the expression of PKC α , P47^{phox}, Nox-2 and phosphorylated ATF-2, as well as intracellular ROS generation, suggesting the inhibitory effect of curcumin on the activation of the PKC α /Nox-2/ROS/ATF-2 pathway. In conclusion, the PKC α /Nox-2/ROS/ATF-2/MMP-9 signaling

pathway is activated in lung cancer A549 cells, which could be modulated by curcumin to inhibit cell invasiveness.

Introduction

As one of the most common malignant diseases of the respiratory system, lung cancer is associated with a 5-year overall survival rate of 15% and is among the leading causes of cancer-related deaths worldwide (1). Treatment failure is mainly caused by the high invasive and metastatic potential, which characterizes its high malignant potential (2). It was reported that ~90% of malignant tumor-related deaths are due to invasion and metastasis, which is also true for lung cancer (3). The mechanisms of invasion of lung cancer are complicated since multiple biological processes such as alterations in gene expression and changes in several signaling pathways are usually involved. Thus, identifying the therapeutic drugs specifically targeting these processes to inhibit the invasiveness of lung cancer is of significant importance.

A zinc-dependent proteinase family, the matrix metalloproteinases (MMPs), are believed to participate in numerous pathological processes due to their activity in extracellular matrix (ECM) degradation (4). MMPs are associated with non-neoplastic diseases including ischemia (5), trauma (6), and neoplastic diseases including glioma (7), and breast (8) and liver cancer (9). It is currently generally accepted that cancer cells invade through tissue by secreting these enzymes. As an important member of the MMPs, MMP-9 overexpression is often detected in various human invasive cancers (10). The regulation of expression of MMP-9 is mediated by multiple activators such as NF- κ B (11). The most direct induction of MMP-9 expression is through the activating transcription factor-2 (ATF-2) (12) which further binds to the AP-1 site of the MMP-9 gene promoter to enhance MMP-9 transcription (13). A previous study found that ATF-2 was activated by phosphorylation in a reactive oxygen species (ROS)-dependent manner in fibroblasts and myocytes (14).

1,7-Bis(4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione, also known as curcumin, is extracted and obtained from the roots of a plant named turmeric (*Curcuma longa* L.).

Correspondence to: Dr Jiansheng Wang, The Second Department of Thoracic Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, P.R. China
E-mail: wangjsh@mail.xjtu.edu.cn

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Curcumin's hydrophobic polyphenol molecular structure contributes to its multiple biological functions including its anticancer activity (15). Studies have revealed that curcumin inhibits malignant cell migration, invasion and metastasis by repressing expression of multiple proteins including MMPs (16,17); however, the exact mechanisms require further investigation.

Curcumin reduces excessive production of ROS in mammalian cells which may lead to multiple biological effects (18). A previous study suggested that cellular ROS were generated in an enzymatic reaction catalyzed by NADPH oxidase-2 (Nox-2) under external stimuli (19). Nox-2 is activated by phosphorylation of its subunit P47^{phox} which was suggested to be phosphorylated by protein kinase C α (PKC α) (20). A recent study pointed out that curcumin inhibits phosphorylation of Nox subunits by reducing PKC expression (21).

In this context, we hypothesized a possible mechanism that curcumin inhibits MMP-9 expression in lung cancer cells by inhibiting activation of PKC α /Nox-2/ATF-2 signaling. The results in the present study broaden the knowledge of the mechanisms of the anticancer activity of curcumin, and also provide new clues for the molecular-targeted treatment of malignant diseases.

Materials and methods

Cell line, culturing and treatment. Human lung cancer cell line A549 was acquired from the Cell Resource Center of the Chinese Academy of Sciences. The cells were cultured in cell culturing flasks (Corning) and maintained in RPMI-1640 culture medium (HyClone) supplemented with fetal bovine serum (FBS, 10%; HyClone), glutamine (2 mmol/l; Sigma) and antibiotics including 100 μ g/ml streptomycin and 100 U/ml penicillin (both from Sigma). Cells were cultured in a cell culture incubator (Thermo) providing a humidified environment with 95% fresh air and 5% carbon dioxide. Cells were treated with serially diluted curcumin (Sigma) at various concentrations (0, 15, 30 and 60 μ mol/l) for 24 h.

Cell viability evaluation. Cell viability of the A549 cells was assessed by colorimetric 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay in accordance with standard methods. After an equal number of cells (2×10^4) were plated in a 96-well plate (Corning), the treated cells were washed with sterilized phosphate-buffered saline (PBS; Pioneer) twice, and then incubated with MTT (Sigma) at a final concentration of 5 mg/ml for 4 h in an incubator. After PBS washing, the cells were dissolved in dimethyl sulfoxide (DMSO; Sigma). The 450-nm absorbance value was read by a plate reader (Bio-Rad). The cell viability was presented as the inhibition rate, which was expressed as a ratio of the number of the non-viable cells in the experimental wells (cells treated by curcumin) compared to the control wells.

DNA and RNA transfection. Six-well plates were seeded with 5×10^4 cells/well in 2 ml media 24 h before transfection; the cells were 80-90% confluent. Cells were transfected with siRNA (100 pmol/well) or plasmid DNA (4 μ g/well) using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. After

48 h of transfection, the cells were collected for subsequent experiments. For establishing stable cell lines, the cells were selected using puromycin for 2 weeks. Stable transductants were pooled. All siRNAs and shRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The pHACE-PKC α WT plasmid (22) was provided by Addgene (Addgene plasmid, 21232).

Cell invasion assay. The invasive ability of the A549 cells was assessed by Transwell assays using the BioCoat Matrigel invasion assay system (BD Biosciences) according to standard protocols. After suspension in serum-free medium, the cells were seeded. The Matrigel was used to coat the upper surface of the Transwell chambers for invasion assay. After a 24-h incubation, the cells that invaded through the membrane were fixed with methanol. The invasion was then observed and evaluated after crystal violet staining under an optical microscopy.

Intracellular ROS detection. The intracellular ROS level in the A549 cells was detected by 2',7'-dichlorofluorescein diacetate (DCFH-DA). The cultured A549 cells were incubated with serum-free RPMI-1640 medium supplemented with DCFH-DA (Beyotime) at a final concentration of 10 μ mol/l at 47°C for 40 min. Then the fluorescence of DCFH-DA at 530 nm was detected using the FACSCalibur flow cytometer (BD Biosciences).

Quantitative real-time PCR. Total RNA from the A549 cells was isolated by using the RNeasy Mini kit (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was then performed using SYBR-Green II according to the PrimeScript RT-PCR kit protocol (Takara). The specific primers for each gene (PKC α , Nox-2, MMP-9 and β -actin) were designed and synthesized by Takara. β -actin was introduced as the internal control. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression levels for PKC α , Nox-2 and MMP-9.

Western blot analysis. The A549 cells in each group were harvested and lysed in RIPA lysis buffering system (pH, 7.5; 40 mmol/l Tris-HCl, 150 mmol/l KCl, 100 mmol/l NaVO₃, 1 mmol/l EDTA, 1% Triton X-100 and 1 mmol/l PMSF). The concentration of the extracted proteins was determined by the BCA kit (Thermo). The same amount of protein (50 μ g) from each group was separated by SDS-polyacrylamide gel (8 or 10%) vertical electrophoresis and then transferred to PVDF membranes. Defatted milk (5% in TBST buffer) was used to block the non-specific binding by incubation with the membranes at 37°C for 1 h. After washing, the membranes were incubated with specific antibodies against PKC α , Nox-2 and P47^{phox} (all from Abcam), p-ATF-2 and ATF-2 (both from Cell Signaling Technology) and MMP-9 and β -actin (both from Santa Cruz Biotechnology) at 4°C for 12 h. After washing, the membranes were then incubated by corresponding horseradish peroxidase secondary antibodies at room temperature for 1 h. Then an enhanced chemiluminescence kit (Amersham) was used to detect the bands. Software Image J was used to perform the densitometric analysis.

Statistical analysis. Data collected in the present study are expressed as the (mean \pm SD). Differences between groups were analyzed using the Student's t-test. $P < 0.05$ was considered to be indicative of a statistically significant result.

Results

Curcumin inhibits the proliferation and invasion of A549 cells. We first evaluated the effects of curcumin on cancer cell proliferation and invasion. The MTT results showed that curcumin inhibited the proliferation of A549 cells in a dose-dependent manner with a significant anti-proliferative effect $>40 \mu\text{mol/l}$ (Fig. 1A). As it was considered that the inhibition of metastasis may be attributed to the tumor growth inhibition, the concentrations which inhibit cell growth were excluded to avoid the cytotoxic effect of curcumin on cell invasion. Thus, a concentration range $<40 \mu\text{mol/l}$ (10, 20 and $30 \mu\text{mol/l}$ particularly in this study) was chosen for subsequent invasion experiments. Transwell assays demonstrated that curcumin inhibited the invasion of the A549 cells at the above concentrations (Fig. 1B).

Curcumin inhibits the invasion of A549 cells by suppressing the expression of MMP-9. The potential involvement of MMP-9 in cellular invasion was evaluated. MMP-9 was effectively upregulated or downregulated by the pcDNA3.1-MMP-9 cDNA plasmid or siRNA (Fig. 2A). As shown in Fig. 2B, cell invasion was significantly increased in the MMP-9-overexpressed cells and reduced in the MMP-9-downregulated cells. We further tested the inhibitory effects of curcumin on both the MMP-9 overexpressed and downregulated A549 cells. As expected, curcumin did not inhibit the invasion of the MMP-9-downregulated A549 cells when compared with the controls (Fig. 2C). However, in the MMP-9-overexpressed A549 cells, curcumin still exhibited an inhibitory effect on cell invasion (Fig. 2C). Furthermore, we also demonstrated that curcumin dose-dependently suppressed the expression of MMP-9 in the A549 cells at both the mRNA and protein levels (Fig. 2D and E), supporting the functional role of MMP-9 in curcumin-inhibited lung cancer cell invasion.

Involvement of PKC α in expression of MMP-9 and cell invasion of A549 cells. The PKC α cDNA plasmid (pHACE-PKC α WT) and siRNA were used to establish the PKC α upregulated and downregulated A549 cells (Fig. 3A). Expression of MMP-9 was then evaluated by real-time PCR and western blot analyses. As shown in Fig. 3B, upregulation of PKC α induced expression of MMP-9 and inhibition of PKC α by siRNA suppressed the MMP-9 protein expression. In addition, we assessed the potential roles of PKC α in the cell invasion of A549 cells. When expression of PKC α was inhibited by siRNA, the number of invasive A549 cells was reduced (Fig. 3C). In contrast, overexpression of PKC α increased the invasive ability of the A549 cells (Fig. 3C).

PKC α -induced expression of MMP-9 is Nox-2-dependent. Since Nox-2 is one of the downstream molecules of PKC α (20,21), we aimed to ascertain whether Nox-2 is involved in the PKC α /MMP-9 signaling pathway. Specific

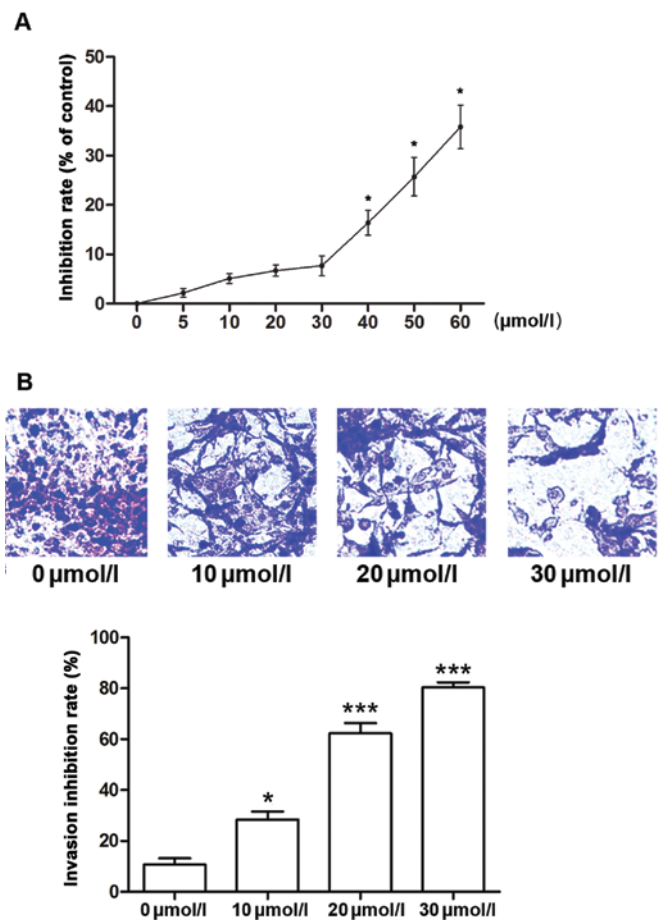


Figure 1. Curcumin inhibits the proliferation and invasion of A549 cells. (A) A549 cells were incubated with curcumin at different concentrations of 0, 5, 10, 20, 30, 40, 50 and $60 \mu\text{mol/l}$ for 24 h. The inhibitory effects of curcumin on the proliferation of A549 cells were assessed by MTT assay. The line graph shows the inhibition rate of values represented as mean \pm SD; * $P < 0.05$. (B) The effect of curcumin on cell invasion in the A549 cells was assessed by Transwell assay. Columns indicate the invasion inhibition rate of curcumin on the A549 cells at different concentrations respectively, * $P < 0.05$, *** $P < 0.001$.

shRNA was used to establish the stable Nox-2-knockdown A549 cells (Fig. 4A). Expression of MMP-9 was then detected in the Nox-2-knockdown and control cells with or without transfection of the PKC α cDNA plasmid. The results showed that knockdown of Nox-2 markedly inhibited PKC α -induced expression of MMP-9 (Fig. 4B). Moreover, we found that overexpression of PKC α increased the expression of P47^{phox} and Nox-2 (Fig. 4C).

PKC α -induced expression of MMP-9 is ATF-2-dependent. We further assessed the potential role of ATF-2 in the PKC α /MMP-9 signaling pathway. Specific shRNA was used to establish stable ATF-2-knockdown A549 cells (Fig. 5A). Expression of MMP-9 was then detected in the ATF-2-knockdown and control cells with or without transfection of the PKC α cDNA plasmid. As shown in Fig. 5B, knockdown of ATF-2 markedly inhibited PKC α -induced expression of MMP-9. Meanwhile, overexpression of PKC α induced phosphorylation of ATF-2 without affecting the protein level of ATF-2 (Fig. 5C).

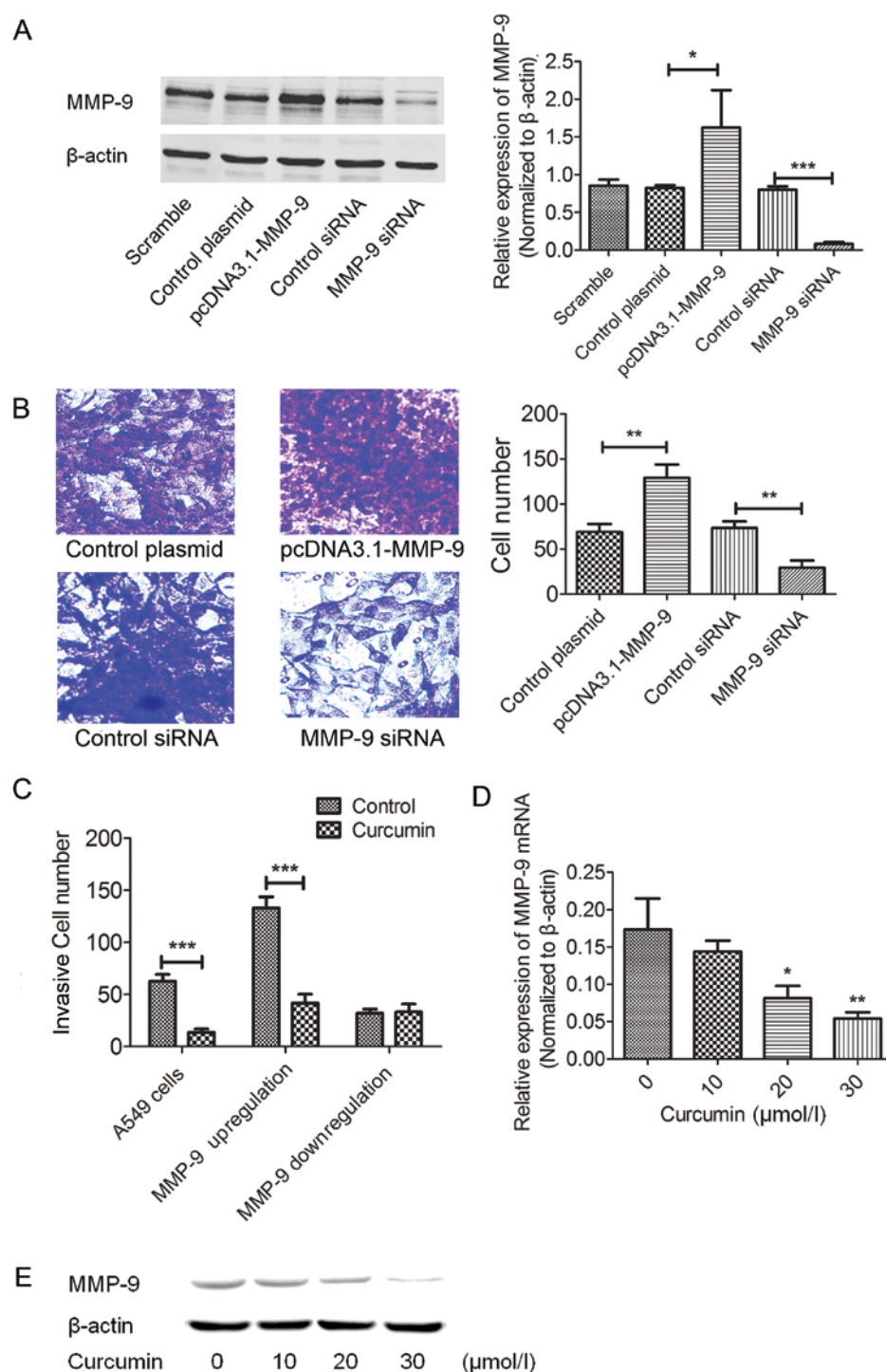


Figure 2. Curcumin inhibits the invasion of A549 cells by suppressing expression of MMP-9. (A) Regulation of MMP-9 expression by cDNA plasmid and siRNA in the A549 cells was detected by western blot analysis; * $P < 0.05$, *** $P < 0.001$. (B) The effect of the regulation of MMP-9 on cell invasion in the A549 cells was determined by Transwell assay. The results are from three independent experiments; ** $P < 0.01$. (C) The effects of the upregulation and downregulation of MMP-9 on cell invasion inhibited by curcumin (30 μ M) in the A549 cells (conducted 48-h post-transfection). The results are from three independent experiments; *** $P < 0.001$. (D and E) A549 cells were treated with the indicated concentrations of curcumin. The expression of mRNA (D) and protein (E) of MMP-9 was detected by real-time PCR and western blot analysis; * $P < 0.05$, ** $P < 0.01$. MMP, matrix metalloproteinase.

Curcumin inhibits activation of the PKC α /Nox-2/ROS/ATF-2 pathway in A549 cells. Western blot analyses were used to assess the expression of PKC α , Nox-2 and ATF-2 in the A549 cells after treatment with curcumin. As shown in Fig. 6A, curcumin significantly inhibited the expression of PKC α , P47^{phox} and Nox-2 in a dose-dependent manner without

affecting the protein levels of ATF-2. However, phosphorylated ATF-2 was markedly reduced by curcumin, suggesting the potential role of curcumin in suppressing the activation of ATF-2. We also detected the intracellular ROS generation in the A549 cells by DCFH-DA fluorescence which was determined by flow cytometry. As shown in Fig. 6B, after

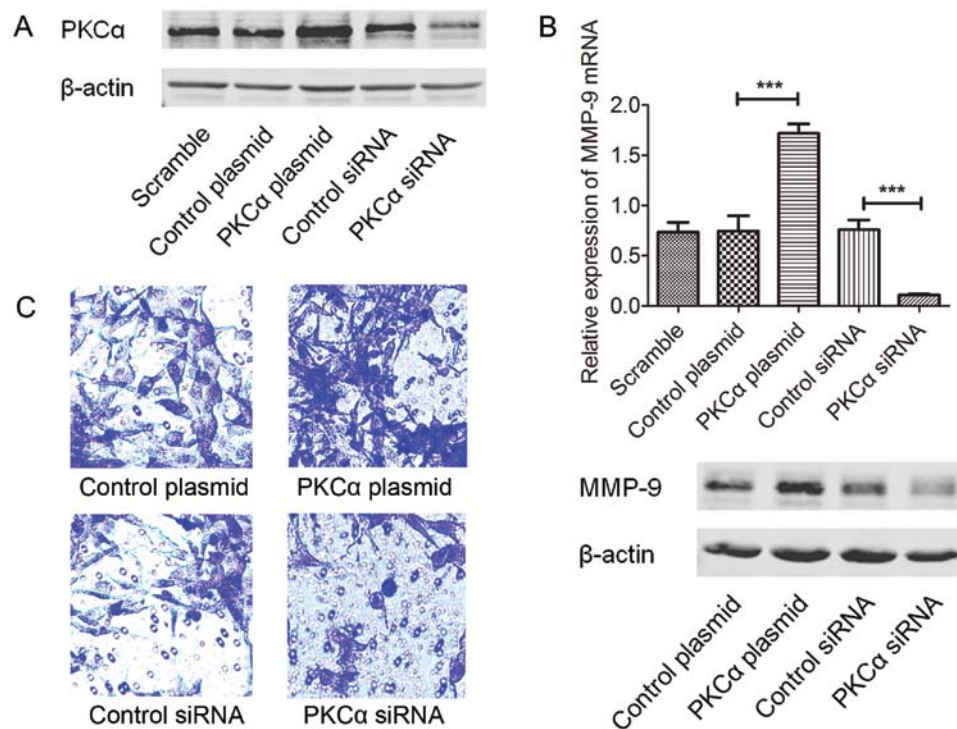


Figure 3. PKCα is involved in expression of MMP-9 and cell invasion in A549 cells. (A) Regulation of PKCα expression by cDNA plasmid and siRNA in the A549 cells was detected by western blot analysis. (B) The effects of upregulation and downregulation of PKCα on expression of MMP-9 in the A549 cells were assessed by real-time PCR and western blot analysis; ***P<0.001. (C) The effects of upregulation and downregulation of PKCα on cell invasion of the A549 cells were assessed by Transwell assay. PKCα, protein kinase Cα; MMP, matrix metalloproteinase.

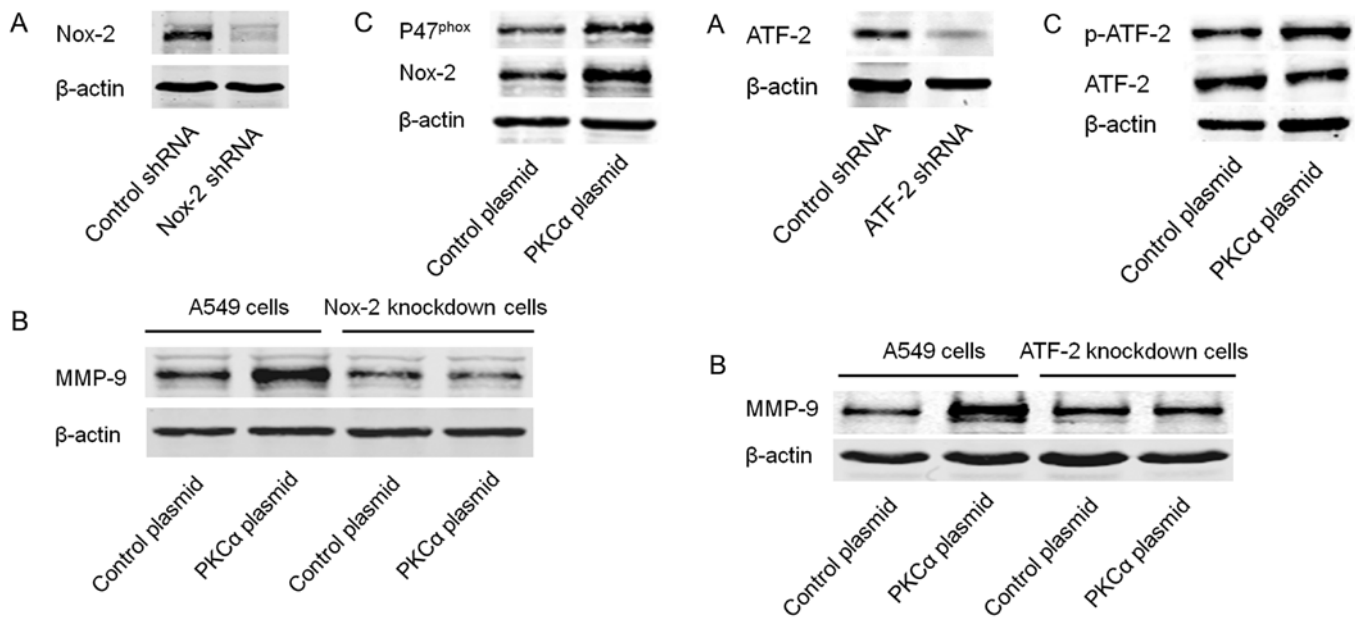


Figure 4. PKCα-induced expression of MMP-9 is dependent on Nox-2. (A) Specific downregulation of Nox-2 by shRNA. (B) The effects of the upregulation of PKCα on expression of MMP-9 in the control and Nox-2-knockdown A549 cells. (C) Upregulation of PKCα-induced expression of P47^{phox} and Nox-2 in the A549 cells. PKCα, protein kinase Cα; MMP, matrix metalloproteinase; Nox-2, NADPH oxidase-2.

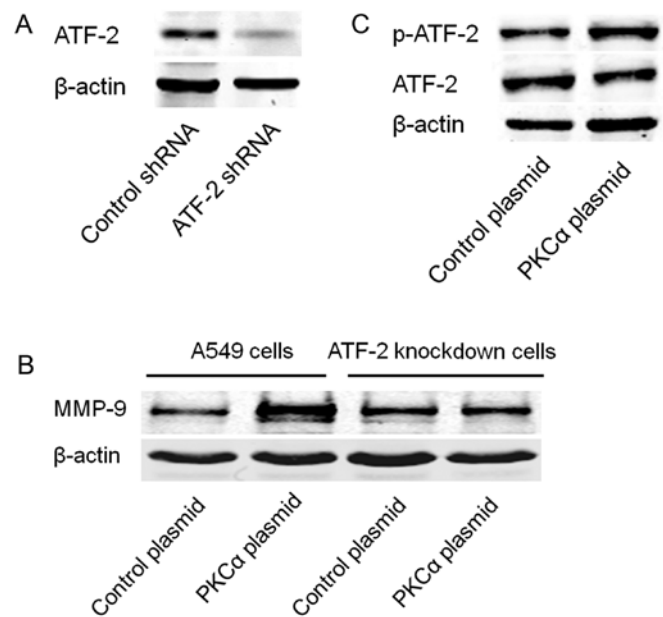


Figure 5. PKCα-induced expression of MMP-9 is ATF-2-dependent. (A) Specific downregulation of ATF-2 by shRNA. (B) The effects of upregulation of PKCα on expression of MMP-9 in the control and ATF-2-knockdown A549 cells. (C) Upregulation of PKCα induced the phosphorylation of ATF-2. PKCα, protein kinase Cα; MMP, matrix metalloproteinase; ATF-2, activating transcription factor-2.

incubation with curcumin, the ROS production was significantly decreased. Similar results of decreased ROS generation were also observed in the PKCα and Nox-2 siRNA-treated A549 cells.

Discussion

Various types of natural therapeutic drugs such as ginsenoside, baicalein and sophocarpine exhibit anticancer effects in

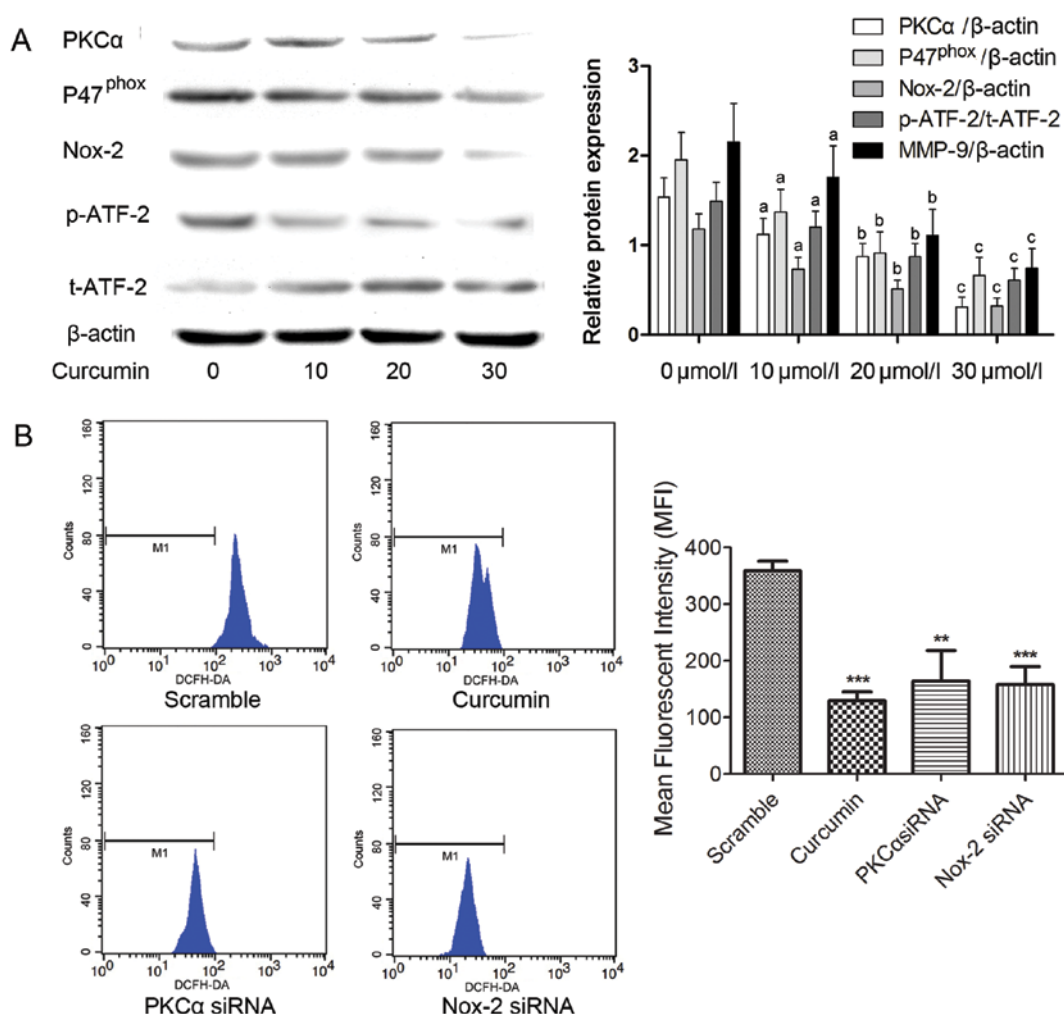


Figure 6. Curcumin inhibits the activation of the PKC α /Nox-2/ROS/ATF-2 signaling pathway in the A549 cells. (A) A549 cells were treated with the indicated concentrations of curcumin. The expression of PKC α , P47^{phox}, Nox-2, phosphorylated (p)-ATF-2 and total (t)-ATF-2 were detected by western blot analysis. The columns indicate the relative protein expression of PKC α , P47^{phox}, (p)-ATF-2 and (t)-ATF-2 in the A549 cells treated with curcumin at the indicated concentrations, respectively. (B) The effects of curcumin (30 $\mu\text{mol/l}$), PKC α siRNA and Nox-2 siRNA on intracellular ROS levels in the A549 cells were assessed by detection of DCFH-DA using a flow cytometer. The columns indicate the mean fluorescent intensity of detected DCFH-DA for each treatment expressed as mean \pm SD of three independent experiments; ** $P < 0.01$, *** $P < 0.001$. PKC α , protein kinase C α ; ATF-2, activating transcription factor-2; Nox-2, NADPH oxidase-2; ROS, reactive oxygen species.

many human types of cancer such as hepatic, breast, cervical and lung cancer (23-25). The therapeutic mechanisms of these natural drugs are extremely complicated, and involve effects on apoptotic-related genes, adhesive molecules and transcription factors (26). In the present study, we demonstrated that the overexpression of MMP-9 which is associated with the invasive ability of lung cancer A549 cells was mediated by the PKC α /Nox-2/ATF-2 signaling pathway. Furthermore, we revealed that the anti-invasive therapeutic effect of curcumin was based on modulating this pathway.

MMPs are a large family of proteases which play crucial roles in the development and progression of cancer (27). As a member of the MMPs, MMP-9 is indispensable in tumorigenesis, invasion and metastasis based on its collagenase activity in ECM degradation (28). Loss of ECM of blood and lymphatic vessel walls prompts cancer cells to spread to organs by invading into blood and lymphatic systems. According to a previous study, the nuclear transcription factor AP-1 regulates the expression of MMP-9 directly by binding to the AP-1 site

of the promoter region of the MMP-9 gene (29). This binding process is believed to depend on ATF-2 which facilitates AP-1 binding to the MMP-9 promoter after forming a heterodimer structure based on its activation by phosphorylation (30). It was reported that the phosphorylation of ATF-2 is induced by excessive intracellular ROS generation (14).

Under many pathological conditions, Nox is the major source of ROS production (31). Nox-2, also regarded as gp91^{phox}, is a prototype NADPH oxidase. As a transmembrane protein, it transfers electrons through a chain reaction to electron donors to form ROS inside cells. The activation of Nox-2 is initiated by phosphorylation of P47^{phox}, the 'organizer subunit', which recruits P67^{phox} and P40^{phox} to form the activated Nox-2 complex (32,33). It has been suggested that the phosphorylation of P47^{phox} is conducted by PKC α in neutrophils (34). Thus, the above described molecules form a signaling pathway which could be referred as PKC α -stimulated Nox-2-dependent ROS-activated ATF-2-induced MMP-9 expression.

In the present study, RNA interference technique was employed to confirm the existence of this pathway in lung cancer cells. Knockdown of both PKC α and Nox-2 not only reduced the production of intracellular production of ROS, but also markedly impaired MMP-9 expression in the lung cancer A549 cells. As a result, PKC α and Nox-2 knockdown suppressed the invasive ability of A549 cells. These results indicate that PKC α /Nox-2/ROS/ATF-2/MMP-9 signaling is activated to induce invasion.

With a long history, from ancient times, curcumin has been used as a herbal remedy in traditional Chinese and Indian medicine in the treatment of numerous diseases (35). In recent decades, studies have identified numerous therapeutic activities including antioxidant, antidiabetic, anti-inflammatory and anticancer for this plant polyphenol, which is extracted from the roots of the spice turmeric (*Curcuma longa* L.) (36,37). Curcumin exerts its inhibitory effects on the invasion and metastasis of many human cancers which are responsible for malignant tumor-related mortality. Several mechanisms of the effects of curcumin have been described in previous studies by investigating MMPs (38), adhesion molecules (39), angiogenesis (40) and the tumor microenvironment (41). We observed that the invasive ability of the A549 cells was significantly inhibited by curcumin treatment via reduced MMP-9 expression. The results also indicated that the MMP-9 reduction was due to curcumin's effect of downregulating the PKC α /Nox-2/ROS/ATF-2 signaling pathway in lung cancer A549 cells.

The PKC α /Nox-2/ROS/ATF-2/MMP-9 signaling pathway is activated in lung cancer A549 cells to maintain invasiveness. Curcumin impairs cell invasion by modulating the PKC α /Nox-2/ROS/ATF-2 signaling pathway to reduce MMP-9 expression in the A549 cells.

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