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ORIGINAL ARTICLE

Shikonin inhibits tumor invasion via down-regulation of NF-κB-mediated MMP-9 expression in human ACC-M cells

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OBJECTIVE: The aim of this study was to examine the anti-invasion effect of Shikonin on human high-metastatic adenoid cystic carcinoma (ACC-M) cells and to explain the possible molecular mechanism involved.

METHODS: The ACC-M cells were treated with Shikonin (0, 2.5, 5, 10 μM) for 24 h. The protein levels and gelatinolytic activities of MMP-2 and MMP-9 were analyzed using Western blot and Gelatin zymography test, respectively. Matrigel invasion assays were used to investigate tumor invasive potential and electromobility shift assays were used to determine the activity of NF-κB.

RESULTS: The invasiveness of ACC-M cells was reduced in a dose dependent manner following 24-h treatment of up to 10 μM of the Shikonin at which concentration no cytotoxicity occurred. The protein levels and gelatinolytic activities of MMP-9 were significantly suppressed by increasing Shikonin concentrations. The down-regulation of MMP-9 appeared to be via the inactivation of NF-κB as the treatment with Shikonin suppressed the protein level of phosphate-IkBa, which was accompanied by a decrease in DNA-binding level of the factor.

CONCLUSIONS: Shikonin inhibits tumor invasion via downregulation of MMP-9 expression in ACC-M cells. Pharmacologic inhibition of the NF-κB-mediated MMP-9 expression by Shikonin might be a powerful treatment option for ACC patients in future.

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Keywords: Shikonin; adenoid cystic carcinoma; tumor invasion; matrix metallopeptidase-9; nuclear factor-κB

Introduction

Adenoid cystic carcinomas (ACCs) are the most common malignant neoplasms of the minor salivary glands. They can also arise in a variety of sites in the head and neck including the major salivary glands, the tracheobronchial tree, the esophagus, as well as in sites outside the head and neck (Spiro, 1986). Typically, ACCs show a characteristic perineural spread with a considerable risk for local relapse and late distant metastases, the latter sometimes occur several years after the first diagnosis with fatal outcome (Huang *et al*, 1997; Sung *et al*, 2003). No effective drugs that are specifically designed for the treatment of this highly metastatic malignancy are available until now. The detailed mechanisms involved in tumor invasion of ACC, however, still remain unknown.

Studies in recent years have shown that certain natural compounds found in plants may be useful as cancer chemopreventive or chemotherapeutic agents (HemaIswarya and Doble, 2006). To develop a more effective chemotherapeutic agent for ACC, we focused our efforts on Shikonin. Shikonin is an active component isolated from the Chinese herbal medicine, Zicao, which has been used for the treatment of macular eruptions, measles, sore-throat, carbuncles, and burns for thousands of years in ancient China (Chen *et al*, 2002).

In previous studies, Shikonin and its derivatives were shown to have anti-proliferative and apoptotic qualities against some tumor cells, including sarcoma 180 (S-180) ascites cells, gastric cancer, colon adenocarcinoma, and oral cancer (Yoon *et al*, 1999; Gaddipati *et al*, 2000; Ruan *et al*, 2008). The signaling involved in Shikonin treatment including blocking epidermal growth factor receptor pathway (Singh *et al*, 2003) in human epidermoid carcinoma cells and inhibiting NAT activity (Yeh *et al*, 2004) in human bladder cancer cells. A recent report has also shown that Shikonin could activate p53 and caspase-9 pathways (Wu *et al*, 2004) in human malignant melanoma A375-S2 cells. However, little is

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known about its anti-invasion effect as well as possible mechanism in human ACC cell lines.

In the present study, we used a highly metastatic ACC cell line, ACC-M, to examine the anti-invasion effect of Shikonin and to explain the possible molecular mechanisms involved. Special emphasis was on NF-κB signal transduction pathway. We aim to provide a better understanding of the related mechanisms and an experimental foundation for the clinical use of Shikonin for the prevention and treatment of ACC in the future.

Materials and methods

Materials and reagents

Shikonin was purchased from Calbiochem Company (La Jolla, CA, USA; Cat. No. 565850) and dissolved in DMSO to a final concentration of 50 mM. Subsequent dilutions were made in culture medium. The same proportion of DMSO/culture medium was added to the controls. The final DMSO content was never above 0.1%. Gelatin was obtained from Sigma (St. Louis, MO, USA) and Matrigel was purchased from Becton Dickinson (Bedford, MA, USA). Monoclonal antibodies to MMP-2 and-9 were purchased from Calbiochem (Boston, MA, USA). Monoclonal antibodies to IκBα and phosphorylated-IκBα were purchased from Santa Cruz Biotechnology Incorporation (Santa Cruz, CA, USA). Monoclonal antibodies to PCNA, p65 and anti-MMP-9 neutralizing monoclonal antibody were also purchased from Santa Cruz Biotechnology Incorporation. CytoSelect™ 96-Well Cell Invasion Assay kit was purchased from Cell Biolabs Incorporation (San Diego, CA, USA). LightShift™ chemiluminescent EMSA kit were purchased from Pierce Biotechnology (Rockford, IL, USA). MMP-2 and-9 ELISA Kits were purchased from Chemicon Corporation (Temecula, CA, USA). Tumor necrosis factor-α (TNF-α) was purchased from R&D Systems, Abingdon, UK.

Cell line and culture

Human adenoid cystic carcinoma cell line (ACC-2) and ACC-M (Gu *et al*, 1999), routinely maintained in our laboratory, were used in this study. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

The anti-proliferative effects of Shikonin against ACC-M cells were assessed using the MTT dye [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] uptake method as described earlier (Hussain *et al*, 2007). ACC-M cells were subcultured in a 96-well plate with 2×10^4 cells per well in 100 μl medium. After 24-h incubation at 37°C, the medium in each well was discarded and replaced with a fresh medium at various concentration levels (0, 2.5, 5, 10 and 20 μM) of Shikonin in a final volume of 200 μl. Cells were then incubated at 37°C for 6, 12, 24, and 48 h, respectively. At the end of incubation, 20 μl of PBS solution containing 5 mg ml^{-1} MTT was added to each well, and further incubated for 4 h. Absorbance of each well was measured using an enzyme-linked immunosorbent assay reader at 490 nm after being dissolved in 200 μl DMSO. All experiments were repeated independently at least three times.

Transwell invasion assays

The procedure reported by Repesh (1998) was followed. The invasion of tumor cells was assessed in transwell chambers with a 6.5-mm-diameter polyvinyl/pyrrolidone-free polycarbonate filter of 8-μm pore size. Each filter was coated with 100 μl of a 1:20 diluted matrigel in cold DMEM to form a thin continuous film on the top of the filter. The number of cells was adjusted to 5×10^8 l^{-1} and a 100 μl aliquot containing 5×10^4cells was added to each of the triplicate wells in DMEM containing 10% FBS. After incubation for 24 h, cells were stained and counted as described above, and the number of cells invading the lower side of the filter was measured for invasive activity.

Cytoselect cell invasion assays

To further investigate if Shikonin holds the character to inhibit ACC-M cell invasion, the CytoSelect™ 96-well Cell Invasion Assay Kit (Ji *et al*, 2007), which provides a robust system for the quantitative determination of cell invasion, was used according to the manufacturer's recommendations (Cell Biolabs). Briefly, ACC-M cells were pretreated with Shikonin with different concentration for 24 h. Basement membranes of Boyden chambers were rehydrated with 300 μl serum-free RPMI, and 2.5×10^6 cells were then seeded into the upper area of the chamber in serum-free RPMI. Bottom wells were filled with RPMI supplemented with 10% FBS containing Shikonin or no Shikonin. After 48 h incubation (37°C, 5% CO_2), non-invasive cells were removed from the upper chamber and cell invasion was assessed using colorimetric analysis. For colorimetric quantification of invasion, inserts were then placed in extraction buffer (200 μl, 10 min), and absorbance at 560 nm was determined after transfer to a 96-well plate (100 μl $well^{-1}$) using a VersaMax microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). To prove that MMP-9 plays roles in ACC-M invasion, anti-MMP-9 neutralizing monoclonal antibody were added to the mixture of ACC-M cells and culture fluid which would directly used in Cytoselect cell invasion assays with the concentration ranged from 0 to 10 μg ml^{-1} (Kossakowska *et al*, 1999; Hu and Ivashkiv, 2006). Serum-free RPMI containing no anti-MMP-9 neutralizing monoclonal antibody was taken as the control.

Western blot analysis

Cells that were treated with 10 μM Shikonin for 24 h were washed with PBS and lysed in a buffer containing 20 mmol l^{-1} Tris–HCl, 150 mmol l^{-1} NaCl, 1% Triton X-100, 1.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $NaVO_3$, 100 mmol l^{-1} NaF, 10% glycerol, 1 mmol l^{-1} EGTA, 10 mmol l^{-1} sodium pyrophosphate, and 1 mmol l^{-1} phenylmethylsulfonyl fluoride, pH 7.5. Cell lysates were

then centrifuged at 12 000 *g* for 15 min at 4°C. The protein concentrations were determined using Bio-Rad protein analysis (Bio-Rad Laboratories, Hercules, CA, USA). After SDS-PAGE, proteins were transferred to PVDF membranes for 2 h at 80 mA. The membrane was blocked in 5% non-fat dry milk overnight at 4°C and then incubated with antibodies against IκBa (1:1000; Santa Cruz), phosphorylated-IκBa (1:1000; Santa Cruz), p65(1:1000; Santa Cruz), PCNA (1:1000; Santa Cruz), MMP-2 (1:1000; Calbiochem), and MMP-9 (1:1000; Calbiochem) for 2 h with constant shaking. The blots were washed three times with TBST followed by treatment with alkaline phosphatase conjugated anti-mouse immunoglobulin G (1:2000 dilutions in PBST) at room temperature for 1 h. The membrane was then washed three times and treated with BCIP/NBT to visualize the proteins. Experiments were repeated at least three times.

ELISA analysis

MMP-2 and -9 secretions were measured using a fluorometric analysis kit according to the manufacturer's recommendations (Chemicon). Briefly, the detached and attached cells were collected, washed with PBS, and lysed in 200 μl of ice-cold cell lysis buffer provided by the manufacturers. The cell extracts were centrifuged, and supernatants were collected. Equal amounts of cell protein extracts (300 μg) were incubated in reaction buffer containing MMP-9 substrate or MMP-2 substrate at 37°C for 2–3 h. The samples were analyzed with a plate reader by the measurement of optical density (OD) at a wavelength of 405 nm. Each experiment was conducted three times.

Gelatin zymography

Protein concentrations in conditioned media were determined using the bicinchoninic acid method (BCA kit) (Pierce, IL, USA). Conditioned media were mixed with an equal volume of 4× sample buffer (200 mM Tris-HCl, 8% SDS, 0.4% bromophenol blue, 40% glycerol), and electrophoresed on 8% SDS polyacrylamide gels containing 2 mg ml^{-1} of gelatin (Sigma). Gels were then washed twice for 30 min in 2.5% Triton X-100 at room temperature, and incubated for 18 h at 37°C in incubation buffer [50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, and 200 mM NaCl]. Gels were then stained for 1 h with 0.25% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) and then destained in destaining buffer (10% acetic acid and 20% methanol).

Nuclear extract and elecrtrophoretic mobility shift analysis

Cells were treated with Shikonin at various concentration (0–10 μM) for 24 h, and nuclear extracts were prepared as described previously (Iimuro *et al*, 1998). Synthetic complementary oligonucleotides were 3′-biotinylated using the biotin 3′-end DNA labeling kit (Pierce) according to the manufacturer's instructions and annealed for 2 h at room temperature. The sequences of the oligonucleotides used are 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ for the wild-type NF-κB. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng μl^{-1} poly(dI-dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, 10 mM EDTA, and 2.5% glycerol in 1× binding buffer (LightShiftTM chemiluminescent EMSA kit; Pierce) using 20 fmol of biotin-end-labeled target DNA and 5 μg of nuclear extract. Unlabeled target DNA (4 pmol) was added per 20 μl of binding reaction where indicated. Analyses were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (HybondTM-N+) in 0.5× Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 120 mJ cm^{-2} and detected using horseradish peroxidase-conjugated streptavidin (LightShiftTM chemiluminescent EMSA kit) according to the manufacturer's instructions.

Statistical analysis

Data were presented as means ± s.d. from at least three independent experiments. Differences between experimental groups and control group were assessed by nonparametric analysis using SPSS software, version 12 (SPSS, Chicago, IL, USA). Statistical significance was defined as $P < 0.05$ for all tests.

Results

Non-cytotoxicity of Shikonin on ACC-M cells

Shikonin was reported to be a chemotherapeutic agent as evidenced by its ability of inhibiting tumor cell proliferation. In this study, we first determined the cytotoxicity of Shikonin by treating ACC-M cells with Shikonin at various concentrations (0, 2.5, 5, 10, and 20 μM) for 24 h. In comparison with that of solvent control (DMSO), the viability of ACC-M cells was not significantly affected by Shikonin, even at a concentration as high as 10 μM (Figure 1). We made the conclusion from the data that a 24-h treatment of

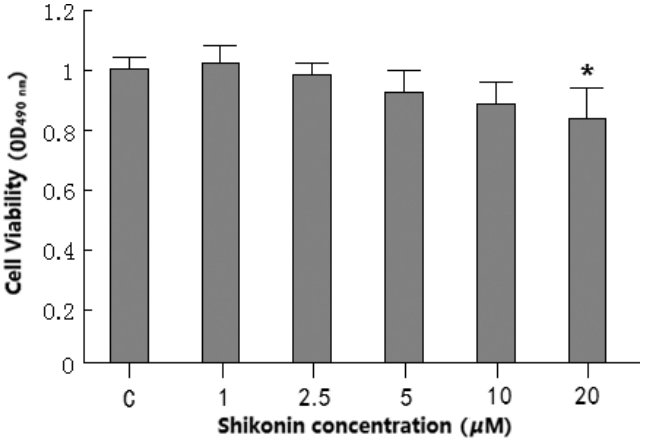

Figure 1 Effects of Shikonin on ACC-M cell viability. MTT assay shows that a 24-h treatment of Shikonin at a concentration ranging from 2.5 to 10 μM has no cytotoxicity on ACC-M cells. The results were presented as the average of three independent experiments ± s.d. *$P < 0.05$ compared with the control

Shikonin at a concentration ranging from 2.5 to 10 μM has no cytotoxicity on ACC-M cells. This concentration range was then applied in all subsequent experiments.

Shikonin inhibit the invasion behavior of ACC-M cells

To examine the effect of Shikonin on the invasive ability of ACC-M cells, transwell invasion assay was used in a dosage experiment. ACC-M cells treated with Shikonin (0, 2.5, 5 and 10 μM) for 24 h were plated in the upper chamber, and the number of cells that moved to the underside of the coated membrane was counted 6 h later under a light microscope. The results showed that the number of cells that invaded the lower chamber was significantly decreased by the 24-h treatment of Shikonin. Such a significant reduction was concentration dependent after treated with 5.0 μM Shikonin (Figure 2a,b). A similarly result was also achieved when CytoSelect™ 96-well Cell Invasion Assay, which provides a robust system for the quantitative determination of cell invasion, was used to further certificate the anti-invasion effect of Shikonin on ACC-M cells (Figure 2c).

Shikonin downregulated the MMP-9 activity in ACC-M cells

As MMP-2 and MMP-9 play a critical role in tumor cell invasiveness, we examined the effect of Shikonin on MMP-2 and MMP-9 expression. As shown in Figure 3a, Shikonin tremendously reduced MMP-9 expression in a concentration-dependent manner, whereas MMP-2 expressions was not affected. And anti-MMP-9 neutralizing monoclonal antibody could significantly inhibit the invasiveness of ACC-M cells which suggest that MMP-9 play an important role in ACC-M invasion (Figure 2d). To further elucidate the down-regulatory effects of Shikonin on MMP-2 and-9, ELISA analysis and Gelatin zymography test were performed to evaluate MMP-2 and-9 secretion and activity respectively. It was revealed that the treatment of 5.0 μM Shikonin for 24 h significantly reduced the secretion level of MMP-9 as well as its *in vitro* activity compared with control, while no significant decrease was shown in MMP-2 (Figure 3b,c). However, in ACC-2 cells, MMP-9 protein expression was much lower than that in ACC-M cells and Shikonin treatment could not inhibit MMP-9 expression in ACC-2 cells significantly (Figure 3d,e).

Shikonin decreased the activity of NF-κB pathway in ACC-M cells

We then test whether Shikonin perturbed the translocation of NF-κB into the nucleus in ACC-M cells by Western blot analysis. Data in Figure 4a demonstrated that the nuclear levels of phosphorylated-IkBa were

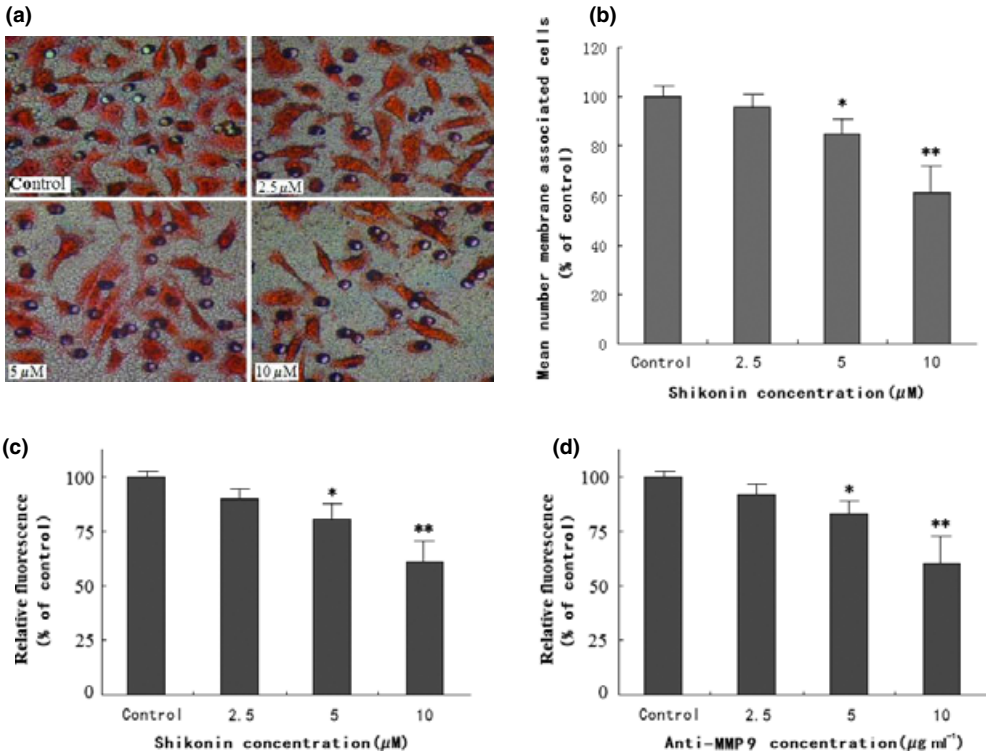

Figure 2 Shikonin impairs the invasiveness of human ACC-M cells. (**a**) ACC-M cells were treated with various concentrations (0–10 μM) of Shikonin for 24 h and detected by Transwell invasion assays. Representative photomicrographs of the membrane-associated cells (jacinth part) were assayed using Eosin staining (×400). (**b**) Semi-quantitative analysis of the anti-invasion effects of Shikonin on ACC-M cells. '% of control' denotes the mean number of the cells expressed as a proportion of the control group and the average of three independent experiments ± s.d. $*P < 0.05$, $**P < 0.01$, compared with the control. (**c**) Colorimetric analysis (absorbance at 560 nm) of Shikonin modulation of ACC-M cell invasiveness with different concentrations (0–10 μM) for 24 h by the CytoSelect cell invasion assay. All data presented as means ± s.d. of three repeats from one independent study $*P < 0.05$; $**P < 0.01$, compared with the control. (**d**) Colorimetric analysis (absorbance at 560 nm) of anti-MMP-9 neutralizing monoclonal antibody on the invasiveness of ACC-M cells with different concentrations (0–10 μg ml^{-1}) for 24 h. All data presented as means ± s.d. of three repeats from one independent study $*P < 0.05$; $**P < 0.01$, compared with the control

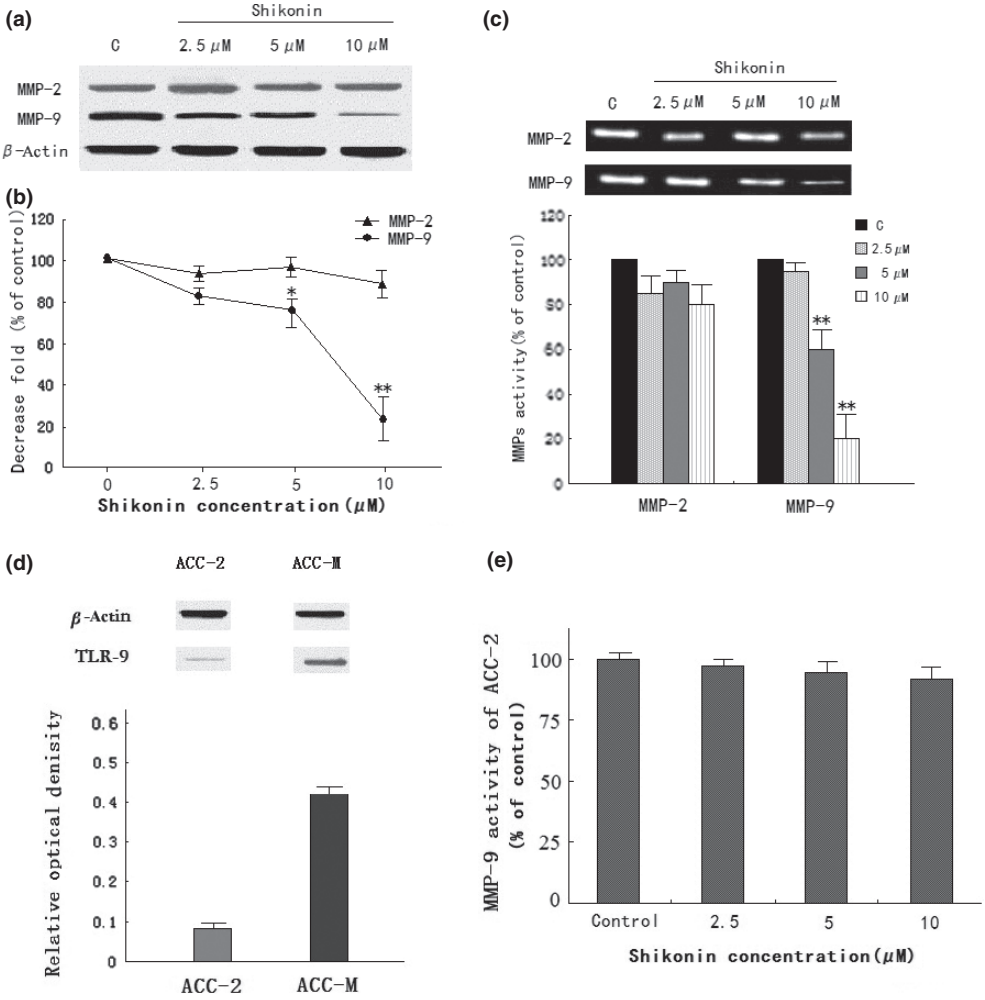

Figure 3 Effects of Shikonin on MMP-2 and MMP-9 expressions. (**a**) Analysis of MMP-2 and -9 protein expression in cells treated with indicated doses of Shikonin and harvested at 24 h. β-actin served as internal control. (**b**) Determined secretion of MMP-2 and -9 was subsequently quantified using ELISA analysis, and presented as means $\pm$ s.d. of three repeats from one independent study. (**c**) ACC-M cells in serum-free medium were treated with various concentrations (0–10 μM) of Shikonin for 24 h. The culture medium of cells after treatment was subjected to gelatin zymography to analyze the activity of MMP-2 and -9; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the control. (**d**) Western blot analysis suggested a low abundance of MMP-9 expression in ACC-2 cells compare with the ACC-M subclone. (**e**) Shikonin treatment could not inhibit the expression of MMP-9 in ACC-2 cells significantly, as revealed by ELISA analysis

significantly decreased after Shikonin treatment in comparison with control. Subsequently, by electrophoretic mobility shift analysis (EMSA), we found high NF-κB DNA-binding activity in untreated ACC-M cell. But when treated with Shikonin, the nuclear NF-κB DNA-binding activity was tremendously decreased, indicating significant inactivation of the NF-κB pathway in ACC-M cells. (Figure 4b,c).

TNF-α-induced MMP-9 expression is inhibited by Shikonin in ACC-M cells

To further elucidate low expression of MMP-9 were regulated by NF-κB pathway, we use TNF-α to stimulate the ACC-M cells and the data show that MMP-9 were highly expressed after 50 μM TNF-α stimulation. When cells were treated in the presence of both Shikonin and 50 μM TNF-α for 24 h, the gelatinolytic activities of MMP-9 were found to be reduced stepwise with increasing concentrations of Shikonin (Figure 5).

Discussion

Recently, there has been significant research interest around Shikonin, a novel compound isolated from the Chinese herbal medicine, Zicao, because of its antitumor effect (Chen *et al*, 2002). However, there is little report to date about its effect on tumor invasion.

In this study, we showed that Shikonin inhibited the invasion of human ACC-M cells *in vitro*, as evidenced by a decreased cell migration and invasion in a dose-dependent manner, especially with the concentration range from 0 to 10 μM. However, Shikonin could directly make the cell to death when the concentration up to 20 μM for 24 h in our preliminary study. We think this cytotoxicity caused by high concentration of Shikonin could also to be understood as another way to inhibit tumor cell invasion. To clearly reflect the anti-invasion effect as well as molecule mechanism involved, ACC-M cells were treated with Shikonin with the

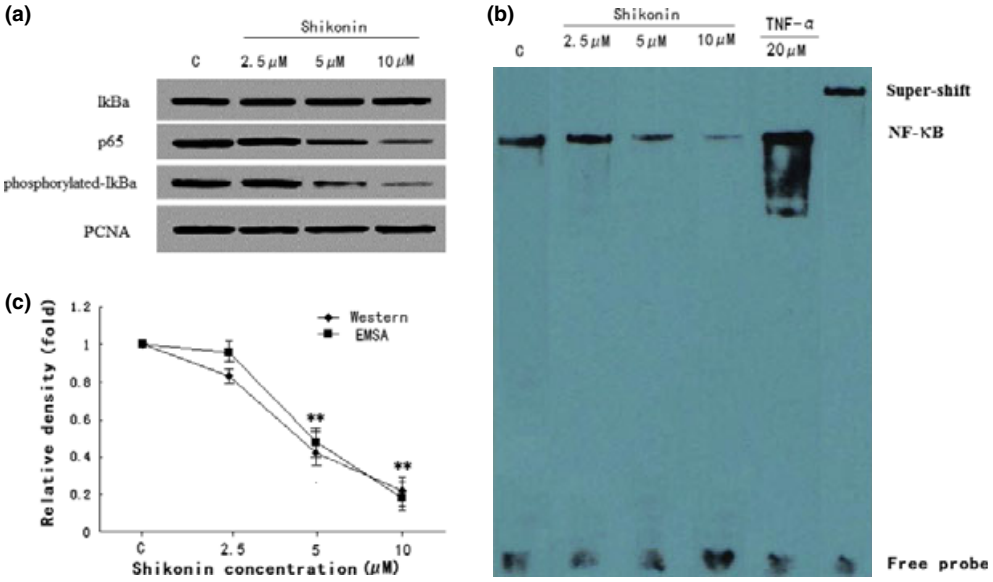

Figure 4 Effects of Shikonin on NF-κB activity. (**a**) Effects of Shikonin on the expression of phosphorylated-IkBa and p65. ACC-M cells were treated with various concent rations of Shikonin for 24 h and the protein levels were determined using Western blot. PCNA served as an internal control. (**b**) Elecrtrophoretic mobility shift analysis (EMSA) analysis of NF-κB in ACC-M cell lines before and after treatment with Shikonin at different concentration for 24 h. (**c**) Determined expression of phosphorylated-IkBa and DNA-binding level were subsequently quantified using densitometric analysis. All data presented as means ± s.d. of three repeats from one independent study $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the control

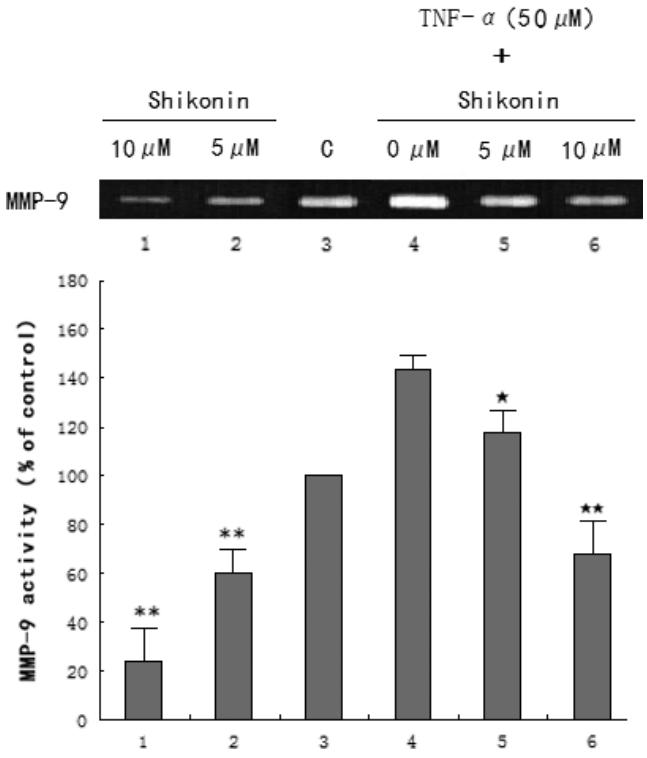

Figure 5 Tumor necrosis factor-α (TNF-α) stimulated MMP-9 expression is inhibited by Shikonin in ACC-M cells. TNF-α stimulation significantly increased the MMP-9 activity, and Shikonin could reverse such effect in a concentration dependent manner. All data presented as means ± s.d. of three repeats from one independent study ($^{*}P < 0.05$; $^{**}P < 0.01$, compared with the panel 3), ($^{*}P < 0.05$; $^{**}P < 0.01$, compared with the panel 4)

concentration range from 0 to 10 μM in subsequent experiments.

The mechanisms underlying such actions, however, are still not clear. Considering that MMPs play a key role in tumor metastasis (Stetler-Stevenson, 2001) and previous reports on high expression of MMP-2 and MMP-9 in ACC-M (Kayano *et al*, 2004; de Vicente *et al*, 2008), we examined the alteration of MMP-2 and MMP-9 expression in Shikonin-treated ACC-M cells. The data show low expression level of MMP-9 in Shikonin-treated ACC-M cells. This suggests that the decrease in the metastasis-associated phenotypes, such as cell migration and cell invasion induced by Shikonin treatment, may be mediated in part by its down-regulation of MMP-9 expression because these effects are highly correlated (Stetler-Stevenson, 2001; Van den Steen *et al*, 2002). Accordingly, the Shikonin-mediated change in the activity levels of MMP-9 coincided well with the protein levels as evidenced by ELISA analysis and Gelatin zymography test, indicating that low activity of MMP-9 were directly caused by low protein synthesis and secretion.

Previous reports have demonstrated that MMP-9 promoter has several transcription factor-binding motifs, including NF-κB (Himelstein *et al*, 1998; Beppu *et al*, 2002). Therefore, the signal transduction pathway of NF-κB may play an important role in the regulation of MMP-9 expression. In fact, in our previous research, we have found much higher activation of NF-κB in ACC-M than that in low-metastatic adenoid cystic carcinoma (ACC-2) (Zhang and Peng, 2007). As a major pathway for tumor invasion, NF-κB can regulate the

molecules that are important in cell migration and invasion, such as ICAM-1, VCAM-1, COX-2, IL-1, IL-8, IL-6, etc. (Pahl, 1999; Rahman *et al*, 1999; Pueyo *et al*, 2000). In addition, this pathway is involved in cell survival/apoptosis, cell growth, cell size regulation as well as angiogenesis (Pikarsky *et al*, 2004; Karin, 2006). Therefore, the inhibition of this pathway is therapeutically very important. Remarkably, here we found a significant inactivation of NF-κB in Shikonin-treated ACC-M cells. Although our study highlights MMP-9 as a target of Shikonin, it is more likely that several other proteins that are involved in invasion are also targets of Shikonin considering the potent function of this towards NF-κB pathway.

To further demonstrate that MMP-9 was regulated by NF-kappa B pathway, we used TNF-α, a special stimulator of NF-kappa B pathway to investigate the expression pattern of MMP-9. Results show that TNF-α could significantly increase the NF-kappa B activity as well as the subsequent MMP-9 expression in a dose dependent manner and that these effects could be partly reversed by Shikonin treatment. Thus, we may draw the conclusion that MMP-9 expression was at least partly regulated by NF-kappa B pathway and NF-kappa B-mediated MMP-9 expression were down-regulated by Shikonin treatment, which may at last lead to the inhibition of ACC-M cell invasion.

In summary, we demonstrated that Shikonin has significant anti-migration and anti-invasion activities against ACC-M cells, and that this effect at least partly through the down-regulation of NF-κB-mediated MMP-9 expression. Therefore, Shikonin could be a potential therapeutic candidate against tumor invasion. Further studies on more detailed mechanisms and functions on Shikonin are needed.

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