Oxymatrine inhibits epithelial-mesenchymal transition through regulation of NF-κB signaling in colorectal cancer cells

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Abstract. Oxymatrine, a traditional Chinese herb extracted from *Sophora flavescens* Ait., displays strong anti-inflammatory and anticancer activities, but how oxymatrine exhibits anticarcinogenic effects in human colorectal cancer (CRC) remains uncertain. The present study aimed to elucidate the exact mechanism by which oxymatrine exhibits anticarcinogenic effects in CRC using the human colon cancer RKO cell line as the experimental model. CRC cells were treated with oxymatrine, and cell proliferation, migration and invasion were examined by colorimetric MTT, Transwell chamber and wound healing assays, respectively. In addition, epithelial-mesenchymal transition (EMT) markers and p65 were assessed by western blot analysis. Our study demonstrated that oxymatrine hindered the proliferation, migration and invasion of the CRC cells. Mechanistically, we found that oxymatrine modulated the expression of EMT markers including E-cadherin, Snail and N-cadherin, and reduced expression of p65 which is crucial to NF-κB activation. In conclusion, our results indicate that oxymatrine reduces the activation of the NF-κB signaling pathway and inhibits CRC invasion by modulating EMT.

Introduction

Colorectal cancer (CRC) is one of the most common carcinomas in the world and the incidence is increasing in Asia (1). Despite many advances in the treatment of CRC, metastasis remains a difficult challenge (2). Although the exact molecular mechanisms are not completely understood, it is well established that epithelial-mesenchymal transition (EMT) is indispensable for carcinoma aggression, development and drug resistance (3). Some researches have demonstrated that tumors undergoing EMT are associated with poor prognosis (4-6). Traditionally, the mechanism(s) controlling the development of cancer are proliferation and apoptosis, Yet, efforts to inhibit the growth and spread of CRC has focused on the effective mechanism(s) of EMT by which carcinoma can rapidly migrate.

EMT refers to the process by which epithelial cells forfeit their polarity, change into interstitial cells and can move easily in the extracellular matrix (7,8). Consequently, epithelial cell markers such as E-cadherin are downregulated, while mesenchymal cell markers such as N-cadherin, vimentin, Snail, and Twist are downregulated (9). The loss or reduction of epithelial marker E-cadherin expression is the paramount feature of EMT (10). E-cadherin expression may be suppressed by transcription factors Snail, Twist and Slug, causing the metastasis of colon tumors (11-13). Overexpression of Snail or Twist and loss of E-cadherin expression play a significant role in the malignant progression of a multitude of epithelial carcinomas, such as gastric, breast and prostate cancer (4-6).

However, recent studies have found that oxymatrine, a traditional Chinese herb extracted from *Sophora flavescens* Ait., exhibits various anticancer activities. It is effectively used for the medical therapy of liver fibrosis, viral hepatitis and autoimmune disease (14-18). In addition, previous studies have demonstrated that oxymatrine has antitumor efficacy in human malignant cancer, associated with the stimulation of cell cycle arrest, apoptosis and downregulation of the Wnt/β-catenin signaling pathway (19-23). However, the possible mechanisms of the antitumor activity of oxymatrine in human CRC are not well elucidated.

In addition, recent studies have shown that oxymatrine can prevent NF-κB nuclear translocation (14), suppress the transcriptional activation of NF-κB in the VEGF signaling pathway and improve intestinal epithelial barrier function via the NF-κB-mediated signaling pathway (24,25). In short, oxymatrine has been shown to be closely associated with the NF-κB signaling pathway. The present study aimed to explore the relationship between the antitumor efficacy of oxymatrine and EMT markers in CRC. We found that oxymatrine impeded EMT by inhibiting NF-κB signaling pathway activation.

Materials and methods

Cell culture and transfection. Human colon cancer RKO, HCT116 and SW480 cell lines were acquired from the

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American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics under a 5% CO_2 humidified atmosphere at 37˚C. The shRNAs targeting p65 (Shanghai GenePharma, China) were diluted in OPTI-MEM (Gibco-BRL, Gaithersburg, MD, USA). According to the manufacturer's instructions, the cells were transfected with a complex of Lipofectamine 2000 (Life Technologies Co. Carlsbad, CA, USA) and shRNA oligonucleotides. The cells were cultured in 6-well plates at a density of $3x10^4$ cells/well.

MTT assay to measure cell proliferation. The cytotoxicity of oxymatrine was evaluated by an MTT assay (Sigma-Aldrich). The cells were cultivated into 96-well plates ($1x10^4$/well) and treated with distinct concentrations of oxymatrine (0, 0.25, 0.5, 0.75 mg/ml) for 24 or 48 h. Cells treated with 0.9% NaCl were the control group. Then MTT (20 μg;2 mg/ml) was added to each well and then incubation was carried out for 4 h at 37˚C, and 100 μl DMSO was added to each well. Finally, cell viability was analyzed by utilizing a microplate reader (Thermo Multiskan MK3; Fermentas, Glen Burnie, MD, USA) at a 490-nm wavelength. The cell growth inhibition rate (%) was calculated using the subsequent equation: Inhibitory rate (%) = $(1 - A_{treatment}/A_{control}) \times 100\%$.

Western blot analysis. RKO cells were cultured overnight, and treated with distinct concentrations of oxymatrine or ammonium pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich, USA) for 24 h. RKO cells treated with 0.9% NaCl were used as the control group. Cells were collected and lysed in cell lysis buffer [150 mM NaCl, 1% sodium deoxycholate, 50 mM Tris (pH 7.5), SDS (sodium dodecyl sulfate 0.1%), 1 mM PMSF, 1% Triton X-100, 1 mM EDTA and 1 mM Na_3VO_4]. Then centrifugation was carried out at 12,000 x g for 15 min at 4˚C, and the protein was collected and evaluated using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein (35 μg) from each sample was separated with 5-10% SDS-PAGE. After addition of 5% nonfat milk, each membrane was incubated overnight at 4˚C with the antibody against GAPDH, P65, E-cadherin, N-cadherin or Snail, and then incubated with peroxidase-conjugated secondary antibody (all from Cell Signaling Technology, Inc., USA). The protein semaphores were detected using an enhanced chemiluminescence kit (Pierce) and exposed to X-ray film.

Wound healing assay. RKO cells were put into a 6-well cell culture plate and grown to 70-80% confluency. Then the cells were scratched using a 1-mm tip. RKO cells were cultured in medium containing 2% serum and oxymatrine (0, 0.25, 0.5, 0.75 mg/ml) for 24 h. Images were acquired at 0 and 24 h after the addition of oxymatrine. The migration distance of the RKO cells was measured under a microscope.

Invasion assay. The invasion of the cells was evaluated with 24-well Transwell chambers (Corning, Tewksbury, MA, USA). Cells ($1.0x10^5$) in 0.2 ml serum-free medium were placed into the upper wells, and 0.6 ml medium with 10% FBS was put in the lower wells. The upper wells were coated with 100 μl 5 μg/ml Matrigel (Corning Costar, Corning, NY, USA). The chambers were incubated in 5% CO_2 air for 24 h at 37˚C. Cells penetrating through the porous membrane were detected by crystal violet staining, and then observed with a light microscope. The numbers of cells were counted in four random fields.

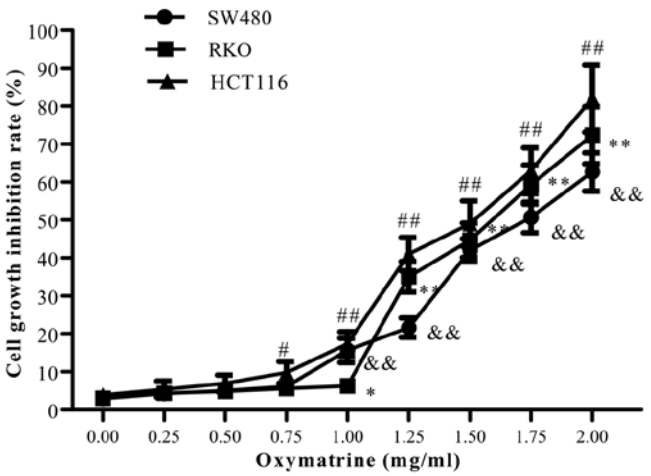

Figure 1. Oxymatrine inhibits the proliferation of CRC cells. SW480, RKO and HCT116 cells were treated with different concentrations of oxymatrine for 24 h, and cell viability was evaluated by MTT assay. Values are presented as means ± SD of three experiments. $^{\&}P<0.05$ and $^{\&\&}P<0.01$, vs. the control group of SW480. $^{*}P<0.05$ and $^{**}P<0.01$, vs. the control group of RKO. $^{\#}P<0.05$ and $^{\#\#}P<0.01$, vs. the control group of HCT116.

Statistical analyses. The experimental data are expressed as mean ± SD, and were statistically analyzed by the t-test method (two-tailed). Statistical data were processed by SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Oxymatrine inhibits the proliferation of CRC cells. To determine the effect of oxymatrine on CRC cell proliferation, SW480, RKO and HCT116 cells were simultaneously treated with various concentrations of oxymatrine (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2 mg/ml) for 24 h. Oxymatrine had no significant effects on the proliferation of CRC cells at a concentration <1 mg/ml. When the concentration was increased to 1 mg/ml, oxymatrine obviously decreased the proliferation of CRC cells in a dose and time-dependent manner (Fig. 1). To exclude the an effect of oxymatrine on cell proliferation, we chose a concentration <1 mg/ml to study the efficacy of oxymatrine on CRC cell invasion in all subsequent experiments.

Oxymatrine suppresses the migratory ability of RKO cells. In order to determine whether oxymatrine suppresses the migratory ability of RKO cells, we performed a wound healing assay. Compared with the control group, the scratch wound in the oxymatrine-treated group healed much slower (Fig. 2A). At 24 h after wounding, the scratch width was 64.00, 39.33 and 28.87% of the control group in the RKO cells following treatment with 0.25, 0.5 and 0.75 mg/ml oxymatrine, respectively (Fig. 2B). These data demonstrated that oxymatrine had the ability to inhibit the migration of RKO cells in a concentration-dependent manner.

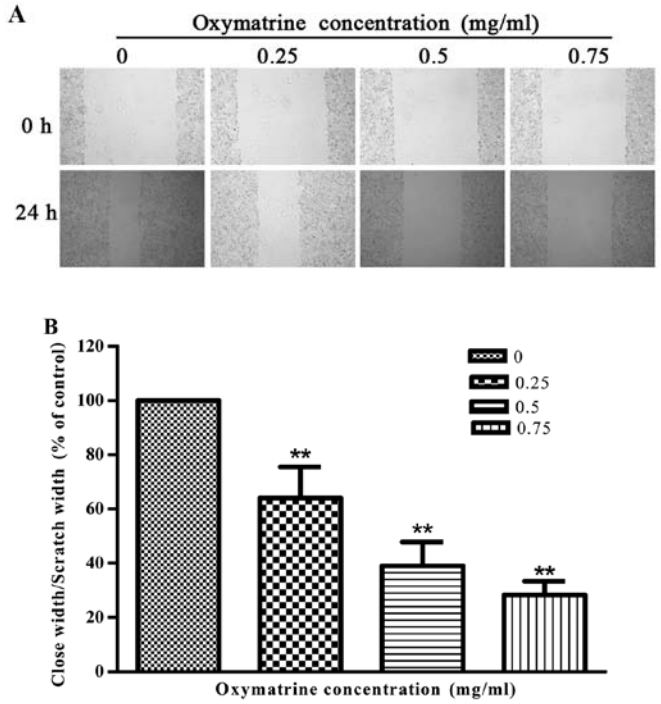

Figure 2. Oxymatrine inhibits the migratory ability of RKO cells. (A) Wound healing assay of RKO cells following treatment with various concentrations of oxymatrine (0, 0.25, 0.5, 0.75 mg/ml). Images were captured at 24 h after wounding (magnification, x100). (B) The migratory ability of RKO cells was expressed as the percentage of the scratch width compared with that noted in the control (0 mg/ml). **P<0.01 vs. the control (0 mg/ml).

Oxymatrine inhibits EMT of RKO cells. To verify whether oxymatrine can inhibit the EMT process in RKO cells, we assessed the protein expression of EMT markers, such as E-cadherin, Snail and N-cadherin, in the RKO cells following treatment with 0.25, 0.5, and 0.75 mg/ml oxymatrine. After 24 h of treatment, the protein levels of N-cadherin and Snail were obviously decreased, however the protein level of epithelial marker E-cadherin was obviously increased in a dose-dependent manner in the RKO cells (Fig. 3).

Oxymatrine inhibits the protein level of p65 in RKO cells. In order to explain the possible mechanism by which oxymatrine inhibits EMT in RKO cells, we further aimed to ascertain which upstream pathway is involved in mediating the effects of oxymatrine. Western blot analysis demonstrated that oxymatrine decreased the protein level of p65 in the RKO cells in a dose-dependent manner (Fig. 4). These data suggest that the NF-κB signaling pathway may mediate the effect of oxymatrine in RKO cells.

Oxymatrine inhibits the invasion of RKO cells via NF-κB signaling. NF-κB signaling is closely correlated with cancer growth and progression. To confirm whether NF-κB signaling mediates the effects of oxymatrine on CRC cell invasion, we inhibited endogenous p65 expression by shRNA method. p65 shRNA significantly inhibited cell invasion. Moreover, p65 knockdown in the RKO cells or the oxymatrine-treated RKO cells caused cellular morphological changes, with the transformation from a long shuttle to cobblestone-like shape and disappearance of tentacles (Fig. 5).

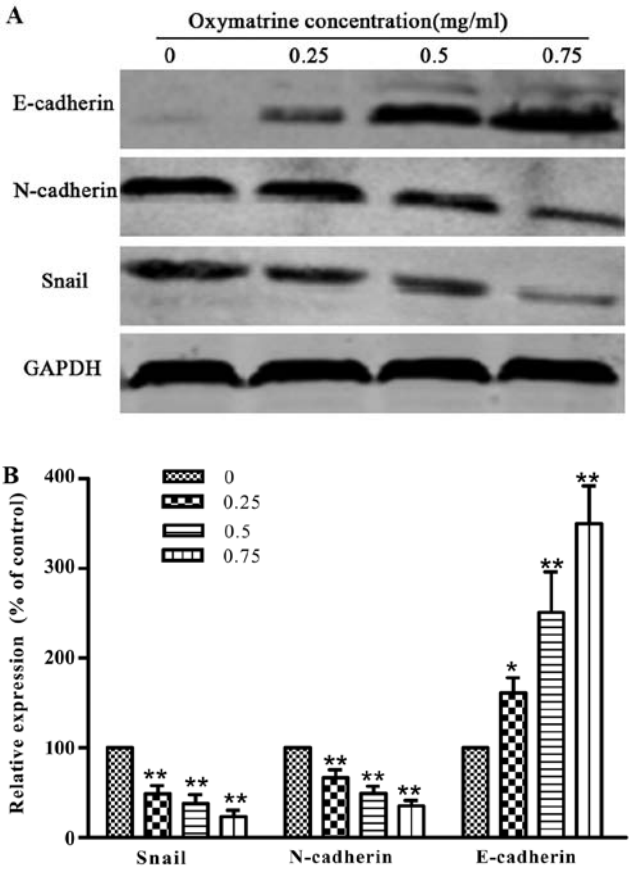

Figure 3. Oxymatrine modulates the expression of EMT markers in RKO cells. (A) RKO cells were treated with oxymatrine (0, 0.25, 0.5 and 0.75 mg/ml) for 24 h, and the protein expression of Snail, N-cadherin and E-cadherin was assessed by western blot analysis. (B) Densitometric analysis of the protein expression of Snail, N-cadherin and E-cadherin in the RKO cells. *P<0.05 and **P<0.01 vs. the control (0 mg/ml).

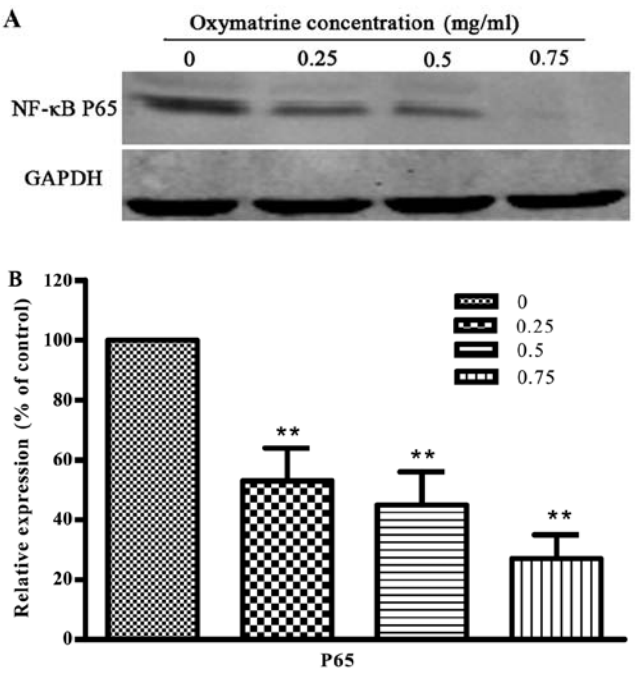

Figure 4. Oxymatrine regulates the p65 signaling pathway in RKO cells. (A) RKO cells were treated with oxymatrine (0,0.25, 0.5 and 0.75 mg/ml) for 24 h, and the protein level of total p65 was detected by western blot analysis. (B) Densitometric analysis of the protein level of total p65 in the RKO cells. **P<0.01 vs. the control (0 mg/ml).

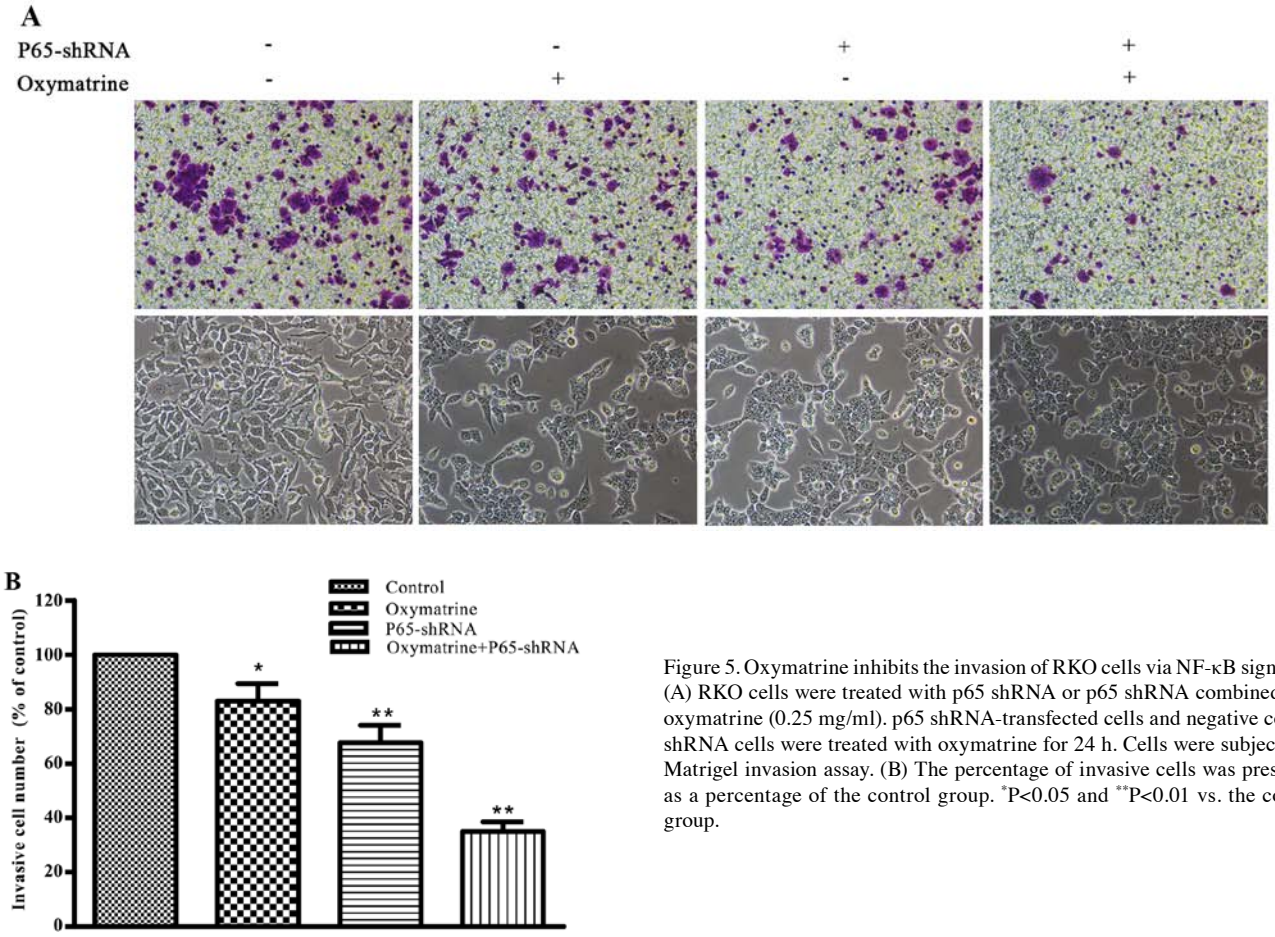

Figure 5. Oxymatrine inhibits the invasion of RKO cells via NF-κB signaling. (A) RKO cells were treated with p65 shRNA or p65 shRNA combined with oxymatrine (0.25 mg/ml). p65 shRNA-transfected cells and negative control shRNA cells were treated with oxymatrine for 24 h. Cells were subjected to Matrigel invasion assay. (B) The percentage of invasive cells was presented as a percentage of the control group. *P<0.05 and **P<0.01 vs. the control group.

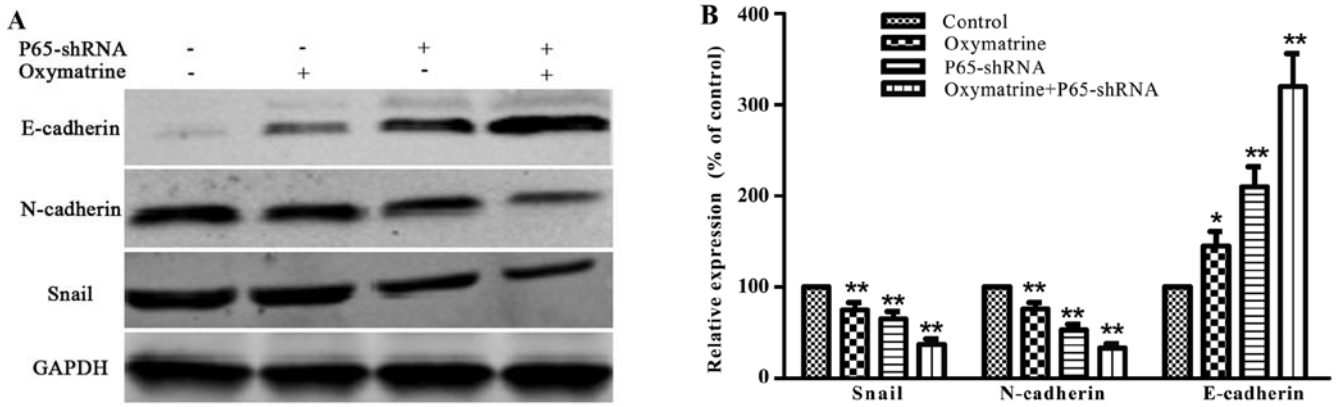

Figure 6. Oxymatrine regulates EMT markers via NF-κB signaling in RKO cells. (A) RKO cells were treated with p65 shRNA or p65 shRNA combined with oxymatrine (0.25 mg/ml). p65 shRNA-transfected cells and negative control shRNA cells were treated with oxymatrine for 24 h. The protein expression levels of Snail, N-cadherin and E-cadherin were assessed by western blot analysis. (B) Densitometric analysis of protein expression levels of Snail, N-cadherin and E-cadherin in RKO cells. *P<0.05 and **P<0.01 vs. the control group.

Oxymatrine regulates EMT markers via the NF-κB signaling pathway in RKO cells. To ascertain whether NF-κB signaling is essential in oxymatrine-mediated expression of EMT markers, we inhibited endogenous p65 expression by shRNA method. E-cadherin expression was obviously increased, however, N-cadherin and Snail were decreased in the p65-shRNA cells (Fig. 6), indicating that p65 is essential for regulation of the EMT process. Simultaneously, we discovered that inhibition of p65 increased E-cadherin expression and decreased expression of N-cadherin and Snail. These data indicate that p65 is essential for the EMT process and EMT inhibition by oxymatrine is partly through the reduction of the activation of the NF-κB signaling pathway.

Discussion

EMT plays a crucial role in carcinoma progression and metastasis. In the present study, we demonstrated that oxymatrine

inhibited the EMT process in RKO cells by decreasing N-cadherin and Snail levels and by increasing the E-cadherin level. The mechanism underlying the efficacy of oxymatrine on EMT in CRC cells was further investigated. We discovered that oxymatrine inhibited the expression of p65, which is important in regulating the expression levels of EMT markers.

Oxymatrine exerts anticancer effects on several types of cancer cells (20-23), but its effects on colorectal carcinoma and its underlying molecular mechanisms remain undetermined. In the present study, we investigated whether a low concentration of oxymatrine exerted an anti-invasive effect on CRC cells. Our results indicated that oxymatrine had the ability to suppress the proliferation of CRC cells at high concentrations (>1 mg/ml). Notably, a low concentration of oxymatrine (<1 mg/ml) exhibited anti-invasive efficacy in the CRC cells. These results indicated that the inhibition of invasion by oxymatrine in RKO cells was not due to cytotoxicity. In addition, we discovered that oxymatrine increased the protein expression level of E-cadherin, an important EMT marker. EMT is a complex process characterized by the loss of epithelial cell-cell adhesion, an important phenotype change in the enhanced invasive ability of cancer cells (26). Snail is considered as one of the major transcription factors which modulate EMT in numerous carcinomas by suppressing E-cadherin (11,27). We further found that oxymatrine inhibited the expression of Snail in RKO cells, suggesting that oxymatrine exerts its anti-invasive effect by inhibiting EMT. To the best of our knowledge, this is the first demonstration of the anti-invasive efficacy of oxymatrine on CRC.

p50/p105, p52/p100, p65, c-Rel, and RelB are 5 members of the NF-κB family, and the NF-κB complex (mainly p65/p50) is maintained in an inactive state by inhibitory IκB protein in the cytoplasm (28). After stimulation, IκB is quickly phosphorylated and degraded via the ubiquitin proteosome pathway. Thus, NF-κB p65 is released into the nucleus and activates a variety of biological processes (29). Oxymatrine prevents NF-κB nuclear translocation (14), suppresses the transcriptional activation of NF-κB in the VEGF signaling pathway and improves intestinal epithelial barrier function via the NF-κB-mediated signaling pathway (24,25). In the present study, we discovered that oxymatrine inhibited the level of p65 in RKO cells, consistent with the above reports. Emerging evidence indicates that NF-κB plays an indispensable role in carcinoma invasion and metastasis (30-32). Moreover, NF-κB can regulate invasion-related genes and is closely linked to invasion and metastasis in CRC (33). Recent studies connect its activity with the regulation of the EMT process. Anoxia/reoxygenation induces EMT in human CRC which may be linked to NF-κB activation (34). We inhibited endogenous p65 expression by shRNA method and demonstrated that oxymatrine inhibited the invasion and modulated EMT in RKO cells at least partially in an NF-κB-dependent manner. These results indicate that oxymatrine may inhibit EMT and invasion via NF-κB signaling in RKO cells. The mechanism underlying the effects of oxymatrine on the NF-κB signaling pathway requires further investigation.

In conclusion, our results indicate that oxymatrine reduces the activation of the NF-κB signaling pathway and inhibits CRC invasion by modulating EMT. Oxymatrine is a promising agent for CRC therapy.

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