

Nitidine chloride suppresses epithelial-to-mesenchymal transition in osteosarcoma cell migration and invasion through Akt/GSK-3 β /Snail signaling pathway

ZHENXIU CHENG¹, YINGLONG GUO², YUBAO YANG³, JINQING KAN⁴, SHIYOU DAI⁵, MENGFEI HELIAN⁶, BO LI⁷, JIA XU⁸ and CHANGYING LIU⁹

¹Department of Neurosurgery, People's Hospital of Linyi City, Linyi, Shandong; Departments of ²Osteology, and ³Joint Surgery, Affiliated Hospital of Shandong Medical College, Linyi, Shandong; ⁴Department of Joint Surgery, People's Hospital of Linyi City, Linyi, Shandong; ⁵Department of Osteology, Qingdao Shili Hospital of Eastern Hospital, Qingdao, Shandong; Departments of ⁶Pathology, and ⁷Cardiology, Central Hospital of Zibo, Zibo, Shandong; ⁸Genetic Disease Diagnosis Center, Zibo Maternal and Child Health Hospital, Affiliated to Shandong Academy of Medical Science, Zibo, Shandong; ⁹Department of Osteology, People's Hospital of Linyi City, Linyi, Shandong, P.R. China

Received February 10, 2016; Accepted March 20, 2016

DOI: 10.3892/or.2016.4846

Abstract. Metastasis is the main cause of death in osteosarcoma. Targeting the process of metastasis is a main strategy for osteosarcoma therapy. As a traditional Chinese medicine, *Zanthoxylum nitidum* (*Roxb*) has been applied to treat various diseases, including cancer. However, no evidence has been shown on the anti-metastasis effect of nitidine chloride (NC) that was extracted from *Zanthoxylum nitidum* (*Roxb*) on osteosarcoma cells, or its underlying mechanisms. In the present study, we aimed to demonstrate the role of NC on the migration and invasion of osteosarcoma cells. Viability and proliferation of osteosarcoma cells were examined by MTT assay. Then, by applying scratch wound healing assay and Transwell assays, we evaluated migratory and invasive ability of the cells, respectively. Moreover, the expression of epithelial-to-mesenchymal transition (EMT) markers were determined after treatment with NC. Furthermore, the expression of Akt, GSK-3 β and Snail were detected by western blot analysis. In addition, the GSK-3 β activity was examined by GSK-3 β kinase assay. Finally, an inhibitor of GSK-3 β , lithium chloride (LiCl) was applied to testify the effect of NC on the expression of EMT markers and Snail. We found that

the proliferative, migratory and invasive ability of the U2OS osteosarcoma cells were all suppressed when treated with NC. NC increased the expression of E-cadherin and decreased the expression of N-cadherin, vimentin and fibronectin in a dose-dependent manner. NC also exerted its ability to suppress the phosphorylation of Akt and GSK-3 β so as to activate GSK-3 β . Then, by using an GSK-3 β inhibitor, LiCl, we revealed the effect of GSK-3 β in the expression of EMT markers. The expression of Snail was inhibited when treated with NC and LiCl also reversed the NC-inhibited Snail expression. Taken together, these results revealed that NC suppressed EMT and decreased the invasive ability of osteosarcoma cells via the Akt/GSK-3 β /Snail signaling pathway.

Introduction

It is well documented that natural products which were extracted from dietary foods or medical plants are reliable sources in chemopreventive or antitumor therapeutic drug pharmaceutical development due to their relatively reduced side-effects (1). Among them, nitidine chloride (NC) (Fig. 1A) is a natural bioactive phytochemical alkaloid which is extracted from the root of *Zanthoxylum nitidum* (*Roxb*). Extensive reports have demonstrated that NC has anti-oxidant, anti-inflammatory, anti-fungal and anti-HIV functions (2,3). Recently, studies have focused on its antitumor activity in various types of cancers, such as hepatocellular carcinoma (4), gastric (5), breast (6,7), renal (8,9), nasopharyngeal carcinoma (10) and chronic myeloid leukemia (CML) (11). NC have showed activity for inhibiting proliferation and induces apoptosis of hepatocellular carcinoma, gastric, breast and renal cancer (4,5,7,8). Moreover, NC was also shown to suppress invasion and metastasis of breast cancer by suppressing c-Src/FAK associated signaling pathway and renal cancer via Akt pathway (6,9). NC has also been demonstrated to be a powerful chemosensitizer for breast cancers (7). Furthermore, a recent study have proved

Correspondence to: Dr Changying Liu, Department of Osteology, People's Hospital of Linyi City, No. 27 Jie Fang Road, Linyi, Shandong 276000, P.R. China
E-mail: liuchangyingsubmit@qq.com

Dr Jia Xu, Genetic Disease Diagnosis Center, Zibo Maternal and Child Health Hospital, Affiliated to Shandong Academy of Medical Science, 12 Xing Yuan Dong Road, Zibo, Shandong 255000, P.R. China
E-mail: jjajia88241@sina.com

Key words: nitidine chloride, epithelial-to-mesenchymal transition, invasion, osteosarcoma cells, GSK-3 β

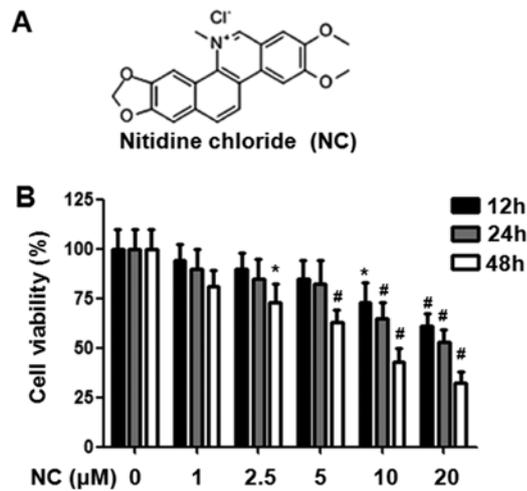


Figure 1. The chemical structure of nitidine chloride (NC) and the effect of NC on U2OS osteosarcoma cell proliferation. (A) Chemical structure of NC. (B) After treated with NC for different time periods (12, 24 and 48 h) at various concentration gradients (0, 1, 2.5, 5, 10 and 20 μM), the viability of the U2OS osteosarcoma cells were detected by MTT assay. * $P < 0.05$, # $P < 0.01$ vs. the control group. The results represent the mean \pm SD from three independent experiments.

that NC can induce erythroid differentiation and apoptosis in CML cells via c-Myc-miRNAs axis (11).

Osteosarcoma is the most common primary bone malignant tumor in young adults and children accounting for approximately 20% of all primary bone neoplasm (12-14). Nowadays, complete radical surgery is a treatment strategy for local osteosarcoma, but the survival rate is below 20%. When chemotherapy is employed with surgery for improving the prognosis, the survival rate is between 50 and 60% (15). Due to distal metastases developed prior to diagnosis, osteosarcoma has a poor prognosis. Evidence showed that less than 30% of patients with osteosarcoma survive for 5 years after lung metastasis (16). The high mortality rate and poor prognosis are attributed to metastasis in osteosarcoma, therefore, it is urgent in osteosarcoma therapy to identify novel drugs to obtain better treatment outcome.

Osteosarcoma cells are highly invasive, so the inhibition of their invasive ability will be effective in its treatment. To gain invasive ability, cancer cells may undergo a unique phenotypic change, epithelial-mesenchymal transition (EMT) which is a morphologic conversion process (17). Characterized by loss of cell adhesion, inhibition of epithelial molecule E-cadherin and gain of mesenchymal markers N-cadherin, vimentin and fibronectin, EMT is considered as a highly conserved cellular process (18). Recent studies have demonstrated EMT in representing mechanisms of carcinoma metastasis (18,19). Among the regulators modulating EMT, Akt and glycogen synthase kinase 3 β (Gsk-3 β), have been shown to play an important role in regulating EMT (20,21). Previous reports have documented the crucial role of GSK-3 β in regulating cell cytoskeleton maintenance, gene transcription, migration and invasion (22-24). Snail family members, including Snail, Slug, Twist, Zeb1 and SIP1, have been demonstrated to play an crucial role in promoting EMT (25).

The present study was designed to investigate the influence of NC on osteosarcoma cell invasion and EMT, as well as

the underlying mechanisms of this effect. To the best of our knowledge, this is the first report to delineate the molecular mechanisms of NC in the invasion and EMT of osteosarcoma cells.

Materials and methods

Cell lines and reagents. The U2OS osteosarcoma cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin in 5% CO_2 at 37°C. Rabbit anti-Akt, phosphor-Akt, rabbit anti-GSK-3 β , phosphor-GSK-3 β and rabbit anti-Snail antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), rabbit anti-E-cadherin, N-cadherin, vimentin and fibronectin antibodies were purchased from Abcam (Cambridge, UK). Alexa-Fluor-488-conjugated and Alexa-Fluor-594-conjugated goat anti-rabbit IgG were purchased from Invitrogen. Nitidine chloride (NC) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China) and then dissolved in dimethyl sulfoxide (DMSO). Lithium chloride (LiCl), a conventional selective inhibitor of GSK-3 β , was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect the cell viability and proliferation. The U2OS osteosarcoma cells (5,000 cells/well) in 100 μl medium were seeded into 96-well plates. After stimulated with NC of various doses (0, 1, 2.5, 5, 10 and 20 μM), for various time-points (12, 24 and 48 h), 20 μl MTT (5 mg/ml) was added into each well. After incubation for 4 h, 100 μl of DMSO was added to each well for another 15 min. Finally, the absorbance values were determined by microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm.

Western blot analysis. After incubated with NC, the U2OS osteosarcoma cells were collected and lysed. Total cell protein concentrations were detected by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal protein from cell lysates were loaded in 12% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) and then blocked with 5% fat-free milk at room temperature for 1 h, and incubated with primary antibodies overnight at 4°C. Then, the membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immune complexes were detected with ECL reagents (Millipore) and the blots were quantified by densitometric analysis using the AlphaImager 2200.

Immunofluorescent staining. After incubated with NC, the U2OS osteosarcoma cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and then incubated with 5% normal goat serum for 1 h. Thereafter, the cells were incubated with rabbit anti-E-cadherin and rabbit anti-vimentin overnight at 4°C. This step was followed by incubation with either Alexa-Fluor-488-conjugated goat anti-rabbit

IgG or Alexa-Fluor-594-conjugated goat anti-rabbit IgG for 1 h at room temperature. The cells were further incubated with 4',6-diamidino-2-phenylindole (DAPI)/PBS (1:5,000; Sigma) for 3 min at room temperature. Finally, images were acquired by using a Nikon Eclipse 80i fluorescence microscope.

Scratch wound healing assay. A scratch wound healing assay was used to examine the migration of the U2OS osteosarcoma cells. Briefly, the U2OS cells (1×10^6 /well) were seeded in 6-well plates cultured with DMEM supplemented with 10% FBS. When reaching confluency, each well was wounded with a scratch by a 200- μ l pipette tip. To determine the effect of NC on the migration of U2OS cells, 5 μ M NC was added to each well. After 24 h of incubation, the wound healing areas were photographed and then the distance between two cell edges were analyzed by ImageJ software.

In vitro invasion assay. To evaluate the effect of NC on the invasive ability of U2OS osteosarcoma cells, the Transwell system was used. The cells were cultured in Boyden chambers, with 8- μ m pore filter inserts, in 24-well plates (Corning Costar, Corning, NY, USA). The pore inserts were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) overnight. The U2OS cells were pre-treated with NC (5 μ M) for 24 h. The U2OS cells (1×10^5 cells/well) were suspended in 100 μ l DMEM supplemented with 1% FBS and were added to the upper chamber. DMEM with 10% FBS and 5 μ M NC were added to the lower chamber. After 24 h of incubation, the cells attaching to the lower surface were fixed with methanol and stained with 0.1% crystal violet. Five random high-power fields (magnification, x200) of each sample were selected and counted to assess the average number of invasive cells.

GSK-3 β kinase assay. A fluorescence peptide substrate-based assay was applied to evaluate GSK-3 β kinase activity (Omnia Ser/Thr Recombinant kit; Invitrogen). In brief, GSK-3 β complex was prepared from equal amounts of cell lysates by immunoprecipitation, then it was incubated with 10 μ M of Ser/Thr peptide substrate in kinase reaction buffer (containing 1 mM ATP and 1 mM DTT) for 20 min at 30°C. Fluorescence intensity was recorded by measuring the A485 in a 96-well plate. Relative GSK-3 β activity was calculated by using untreated cells (equal to 1).

Statistical analysis. The data are expressed as the mean \pm standard deviation (SD). All of the experiments were repeated at least three times. Comparisons among values for all groups were performed by one-way analysis of variance (ANOVA). Holm's t-test was applied for analysis of differences between the different groups. Differences were considered to be statistically significant at $P < 0.05$.

Results

Suppressive effect of NC on the cell proliferation of osteosarcoma cells. The structure of NC is shown in Fig. 1A. To explore the effect of NC on the proliferation of U2OS osteosarcoma cells, MTT assay was used as described in Materials and methods. As shown in Fig. 1B, different doses (0, 1, 2.5, 5, 10 and 20 μ M) of NC were added into the cultured U2OS

osteosarcoma cells. After 24-h treatment, NC significantly inhibited the viability of U2OS osteosarcoma cells at a dose beyond 5 μ M (10 and 20 μ M). After 48-h treatment, NC significantly suppressed proliferation of U2OS osteosarcoma cells at a dose beyond 1 μ M (2.5, 5, 10 and 20 μ M). However, at doses < 1 μ M, the suppressive effect was not significant. Thus, NC suppressed the proliferation of U2OS osteosarcoma cells time- and dose-dependently. Based on these results, we chose NC at a dose of 5 μ M for 24 h in the following migration and invasion experiments so as to exclude the effect of U2OS osteosarcoma cell proliferation.

Migration and invasion are inhibited by NC in osteosarcoma cells. The wound healing assay was used to examine the role of NC on the migration of U2OS osteosarcoma cells. The U2OS osteosarcoma cells were treated with NC at the dose of 5 μ M for 24 h. As shown in Fig. 2A, migration of U2OS cells was inhibited by 5 μ M NC. The results of the wound healing assay indicated that healing over the scratch was significantly reduced when treatment with NC. To further reveal the effect of NC on U2OS osteosarcoma cells invasion, a Transwell assay was applied. The U2OS cells were stimulated with 5 μ M NC for 24 h. As shown in Fig. 2B, the invasive ability of U2OS cells were inhibited by 5 μ M NC. The results of Transwell assay implied that NC could suppress the invasive ability of osteosarcoma cells.

NC alters the expression of EMT markers in osteosarcoma cells. Increasing evidence has demonstrated that EMT played a crucial role in the process of tumour invasion and metastasis (26,27). We thus detected the effect of NC on the expression of EMT markers (E-cadherin, N-cadherin, vimentin and fibronectin) in the U2OS osteosarcoma cells. Various concentrations of NC (0, 5 and 10 μ M) were respectively added to the U2OS cells for 24 h. As shown in Fig. 3A and B, NC treatment significantly upregulated the expression of epithelial marker E-cadherin and downregulated the expression of mesenchymal markers N-cadherin, vimentin and fibronectin in the U2OS cells. Immunofluorescent staining was also applied to evaluate the expression of EMT markers. After NC (0, 5 and 10 μ M) treatment for 24 h, the cells were stained with E-cadherin and vimentin and analyzed by fluorescence microscopy. As shown in Fig. 3C, 10 and 20 μ M NC effectively increased the expression of E-cadherin and decreased the expression of vimentin. These results above suggested that NC may alter the expression of EMT markers in a dose-dependent manner. In addition, these results also suggested that the suppressive effect of NC on U2OS cell invasion and migration may be related to EMT.

Akt/GSK-3 β pathway is involved in the NC modulated EMT in osteosarcoma cells. Akt is a key regulator of cancer development (28). GSK-3 β , known to be a survival factor for cancer, plays an important role in various cancer (29-31). Regulation of EMT through Akt/GSK-3 β signaling pathway has been demonstrated in tumour progression (32,33). To explore the effect of NC on Akt and GSK-3 β activation, we treated the U2OS cells with various concentrations of NC (0, 5 and 10 μ M) for 24 h. Then the proteins were extracted, followed by the detection of the phosphorylation of Akt and GSK-3 β . As

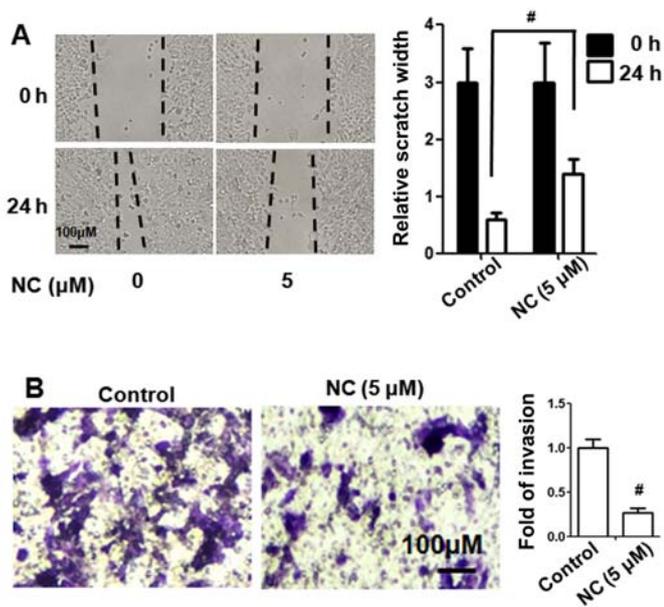


Figure 2. NC inhibits the migration and invasion of U2OS osteosarcoma cells. (A) The U2OS osteosarcoma cells were treated with NC at the dose of 5 μ M for 24 h and then assessed by a wound healing assay. (B) The U2OS osteosarcoma cells were treated with NC at the dose of 5 μ M for 24 h. The effect of NC on invasion of U2OS osteosarcoma cells was detected by a Transwell assay. The bar graph represents the results of three independent experiments. # P <0.01 vs. the control group.

shown in Fig. 4A and B, the relative expression of phospho-Akt and phospho-GSK-3 β was significantly downregulated dose-dependently. To further verify the result, the effect of NC on GSK-3 β activity was examined by GSK-3 β kinase assay. The result demonstrated that NC at various doses (0, 5 and 10 μ M) can significantly enhance GSK-3 β activity in U2OS cells. To further investigate whether the role of NC on EMT was attributed to Akt/GSK-3 β signaling pathway, LiCl, a GSK-3 β inhibitor, was applied to pretreat the U2OS cells for 1 h before NC to block GSK-3 β activation. As shown in Fig. 4D and E, blockade of GSK-3 β activity with LiCl could significantly reverse NC-induced E-cadherin, N-cadherin, vimentin and fibronectin expression. These above results indicate that NC could inhibit Akt phosphorylation and then enhanced GSK-3 β activity. Furthermore, these results suggested that GSK-3 β activation is critical for EMT and Akt/GSK-3 β pathway may be involved in NC-inhibited EMT in osteosarcoma cells.

NC induces Snail upregulation via GSK-3 β activation in osteosarcoma cells. Evidence has shown that EMT is governed by various regulatory networks. Among them, Snail was the first described transcriptional factor in suppressing E-cadherin expression (34). Thus, we further investigated the effects of NC on the expression of Snail and the relationship between GSK-3 β and Snail. We chose various concentrations of NC (0, 5 and 10 μ M) to stimulate the U2OS cells for 24 h. As shown in Fig. 5A and B, NC obviously inhibits the expression of Snail with a dose-dependent manner. Then, the U2OS cells were also pretreated with LiCl for 1 h before NC was added. As shown in Fig. 5C and D, pretreatment with the GSK-3 β inhibitor LiCl significantly abolished the inhibitory effect of

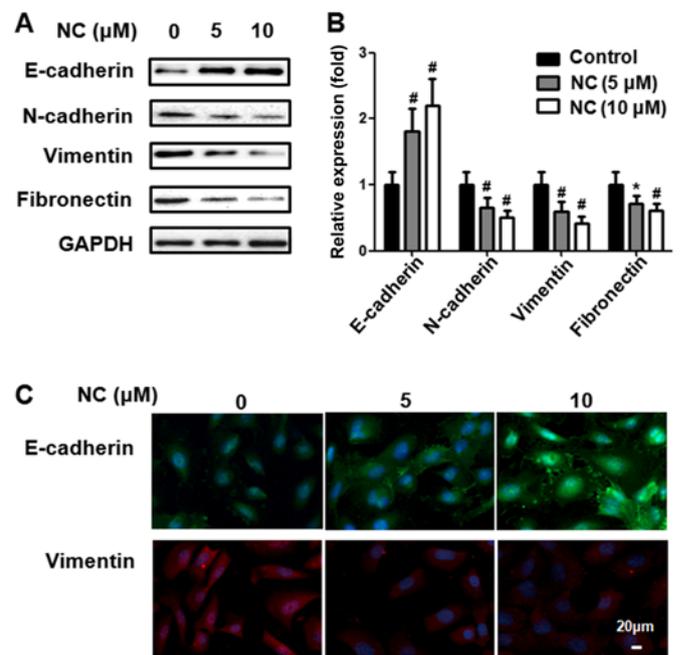


Figure 3. NC increases the expression of E-cadherin and reduces the expression of N-cadherin, vimentin and fibronectin of U2OS osteosarcoma cells. (A) The U2OS osteosarcoma cells were treated with NC at various concentrations (0, 5 and 10 μ M) for 24 h and the level of E-cadherin, N-cadherin, vimentin and fibronectin protein expression were detected by western blot analysis. (B) Statistical analysis of the western blot results. (C) The U2OS osteosarcoma cells were treated with NC at various concentrations (0, 5 and 10 μ M) for 24 h and the expression of E-cadherin and vimentin were detected by immunofluorescent staining. * P <0.05, # P <0.01 vs. the control group. Data shown are means \pm SD from three independent experiments.

NC. These results indicate that GSK-3 β is a key regulator of Snail and NC inhibits the expression of Snail through blocking the GSK-3 β pathway at least in part. Taken together, these results strengthen the opinion that inhibition of metastasis by NC is regulated by suppression of EMT through Akt/GSK-3 β /Snail signaling pathway in osteosarcoma cells.

Discussion

Metastasis is regarded as a leading cause of mortality of most cancer patients. Exploration on the molecular mechanisms of metastasis is a crucial issue in cancer research. Thus, it is very important to seek effective drugs to suppress the metastasis of cancer. Recently, increased attention was paid on natural products in tumor therapy for their anticancer property and less severe side-effects (35,36). As a kind of bioactive phytochemical alkaloid extracted from *Zanthoxylum nitidum* (Roxb), NC has been well documented to have the ability to induce apoptosis and inhibit the proliferation, migration and invasion of hepatocellular carcinoma, gastric, breast, renal cancer cells and CML cells (4-9,11). Although previous studies reported the antitumor effect of NC, the role of NC on osteosarcoma cell migration and invasion have not been clearly elucidated, or EMT and its detailed molecular mechanisms. Our study first examined the effect of NC on the viability of osteosarcoma cells. We found that 5 μ M NC could significantly inhibit cell proliferation after 48 h of treatment, while there was no significant effect before 24 h. Thus, we selected 24 h as the

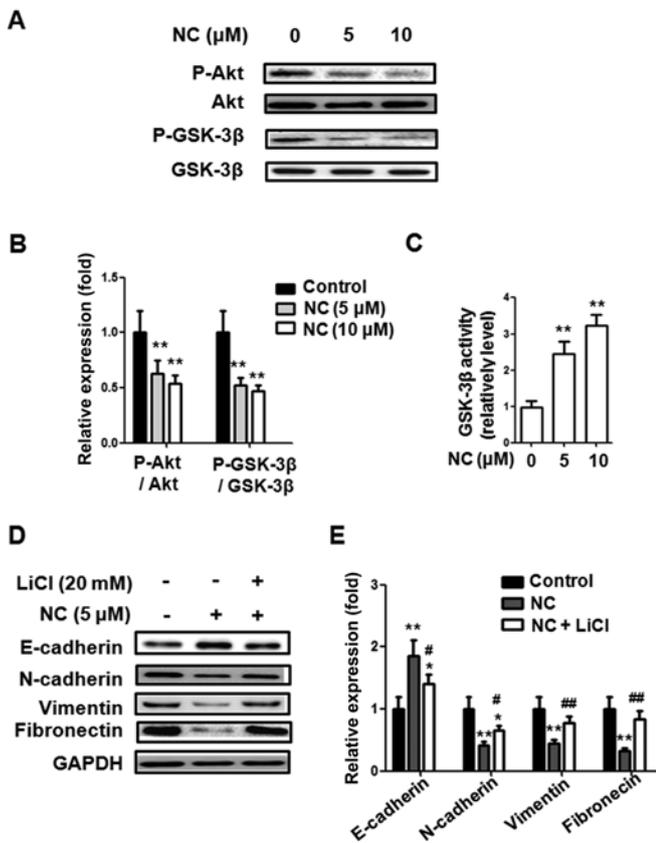


Figure 4. GSK-3β regulates the NC-mediated epithelial-to-mesenchymal transition (EMT) in U2OS osteosarcoma cells. (A) The U2OS osteosarcoma cells were stimulated with NC at various concentrations (0, 5 and 10 μM) for 24 h, expression of p-Akt and p-GSK-3β were analyzed by western blotting. (B) Statistical analysis of the western blotting results. (C) The U2OS osteosarcoma cells were stimulated with NC at various concentrations (0, 5 and 10 μM) for 24 h, GSK-3β activity was determined by GSK-3β kinase assay. (D) The U2OS osteosarcoma cells were treated with NC (5 μM) and/or LiCl (20 mM) for 24 h, then expression of E-cadherin, N-cadherin, vimentin and fibronectin were assessed by western blotting assay. (E) Statistical analysis of the western blotting results. *P<0.05, **P<0.01 vs. the control group. #P<0.05, ##P<0.01, compared with the cells treated with NC. Data shown are means ± SD from three independent experiments.

stimulation time-point and 5 μM as the stimulation dose in the following migration and invasion experiments, so that the influence of proliferation was excluded. For the first time, the present study demonstrated that NC could effectively inhibit the migration and invasion of osteosarcoma cells by using wound healing and Transwell assays. These results revealed the anti-metastatic activity of NC.

The metastasis of osteosarcoma is a complex process which includes cell migration, invasion, and adhesion (37). EMT is a special phenotypic transformation by which epithelial cells lose the polarity and cell-cell adhesion, then obtain the migratory and invasive properties of mesenchymal cells. EMT is characterized by downregulated epithelial marker E-cadherin and upregulated mesenchymal markers N-cadherin, vimentin and fibronectin. Cancer cells can gain the capacity to invade adjacent tissues, break through the basement membrane, and finally enter the bloodstream when acquiring the mesenchymal phenotype through EMT (38). Several studies have reported the role of EMT in osteosarcoma metastasis, thus implying the poor prognosis of

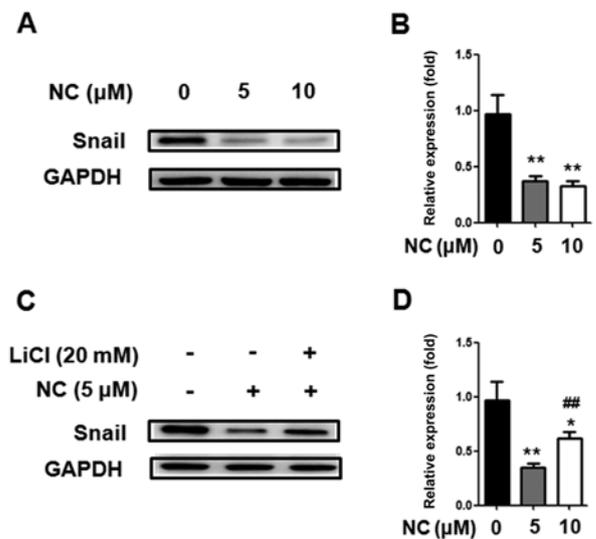


Figure 5. GSK-3β mediates the expression of Snail in NC-treated U2OS osteosarcoma cells. (A) The U2OS osteosarcoma cells were stimulated with NC at various concentrations (0, 5 and 10 μM) for 24 h, expression of Snail were analyzed by western blotting. (B) Statistical analysis of the western blot results. (C) The U2OS osteosarcoma cells were treated with NC (5 μM) and/or LiCl (20 mM) for 24 h, then expression of Snail were assessed by western blotting assay. (D) Statistical analysis of the western blot results. *P<0.05, **P<0.01 vs. the control group. #P<0.05, ##P<0.01, compared with the cells treated with NC. Data shown are means ± SD from three independent experiments.

osteosarcoma (39,40). In this study, we explored the role of NC on EMT in osteosarcoma cells. The present study showed that NC increased the expression of the epithelial marker E-cadherin and decreased the expression of mesenchymal markers N-cadherin, vimentin and fibronectin with a dose-dependent manner. Our results demonstrated that NC inhibited EMT in osteosarcoma cells.

The Akt/GSK-3β signaling pathway has been documented to regulate various cell functions, such as proliferation, apoptosis and angiogenesis (41-43). Expression of Akt/GSK-3β is associated with cell proliferation, apoptosis and angiogenesis (24,41-43). Notably, GSK-3β activity is regulated by a site-specific phosphorylation of Tyr216/Ser9 residues, the phosphorylation of GSK-3β resulted in its inactivation (44). Moreover, Akt/GSK-3β regulates the stability and transcription of Snail in some cancer cells and the inactivation of GSK-3β is crucial to induce Snail expression (32,45). Previous studies documented the role of Akt/GSK-3β/Snail in regulating the metastasis of various type of tumours via modulating EMT, such as colorectal, gastric, hepatocellular carcinoma, prostate, lung, breast, head and neck squamous cell carcinoma and bladder cancer (21,32,33,45-49). In the present study, the Akt/GSK-3β activity in osteosarcoma cells was examined by western blot analysis and GSK-3β kinase assay. We found that expression of phospho-Akt and phospho-GSK-3β was decreased while GSK-3β activity was increased when treated with NC. To confirm that the GSK-3β signaling pathway was involved in NC-inhibited EMT in osteosarcoma cells, we blocked GSK-3β activity by applying LiCl, the GSK-3β inhibitor. The present study showed that suppression of GSK-3β activity by LiCl significantly reversed NC-induced E-cadherin, N-cadherin, vimentin and fibro-

nectin expression. These results further demonstrated that GSK-3 β activation is essential for EMT and the effect of NC might be GSK-3 β -dependent.

Finally, we explored the role of NC and GSK-3 β on transcription factor Snail. Snail, a zinc finger protein, is one of the most crucial members in Snail superfamily. Previous studies have demonstrated its role in modulating EMT through down-regulate the expression of epithelial marker E-cadherin and upregulate of mesenchymal markers N-cadherin, vimentin and fibronectin by binding with some boxes in the promotor region (50). Snail is involved in several types of cancer progression and metastasis via regulating EMT (33,47,50). The stability and transcription of Snail may be modulated by GSK-3 β (32). In the present study, we found that the expression of Snail was decreased when treated with NC in a dose-dependent manner. While LiCl significantly reversed the effect further indicating that GSK-3 β was upstream of Snail. The above results demonstrated that NC inhibited the expression of Snail via the GSK-3 β pathway.

In summary, this study for the first time indicate that NC inhibited the migration and invasion of osteosarcoma cells by suppressing EMT. Moreover, the inhibitory role of NC in EMT was regulated via the Akt/GSK-3 β /Snail pathway. Taken together, NC suppresses the invasive ability of osteosarcoma cells by inhibiting EMT through the Akt/GSK-3 β /Snail signaling pathway. Thus, our finding suggested that NC is a promising agent in osteosarcoma therapy. Further *in vivo* research should be performed.

Acknowledgements

The present study was supported by the Science Foundation of Shandong Province (no. ZR2014HP005).

References

- Gordaliza M: Natural products as leads to anticancer drugs. *Clinical & Translational Oncology: Official Publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* 9: 767-776, 2007.
- Wang Z, Jiang W, Zhang Z, Qian M and Du B: Nitidine chloride inhibits LPS-induced inflammatory cytokines production via MAPK and NF-kappaB pathway in RAW 264.7 cells. *J Ethnopharmacol* 144: 145-150, 2012.
- Del Poeta M, Chen SF, Von Hoff D, Dykstra CC, Wani MC, Manikumar G, Heitman J, Wall ME and Perfect JR: Comparison of *in vitro* activities of camptothecin and nitidine derivatives against fungal and cancer cells. *Antimicrob Agents Chemother* 43: 2862-2868, 1999.
- Liao J, Xu T, Zheng JX, Lin JM, Cai QY, Yu DB and Peng J: Nitidine chloride inhibits hepatocellular carcinoma cell growth *in vivo* through the suppression of the JAK1/STAT3 signaling pathway. *Int J Mol Med* 32: 79-84, 2013.
- Chen J, Wang J, Lin L, He L, Wu Y, Zhang L, Yi Z, Chen Y, Pang X and Liu M: Inhibition of STAT3 signaling pathway by nitidine chloride suppressed the angiogenesis and growth of human gastric cancer. *Mol Cancer Ther* 11: 277-287, 2012.
- Pan X, Han H, Wang L, Yang L, Li R, Li Z, Liu J, Zhao Q, Qian M, Liu M, *et al*: Nitidine chloride inhibits breast cancer cells migration and invasion by suppressing c-Src/FAK associated signaling pathway. *Cancer Lett* 313: 181-191, 2011.
- Sun M, Zhang N, Wang X, Cai C, Cun J, Li Y, Lv S and Yang Q: Nitidine chloride induces apoptosis, cell cycle arrest, and synergistic cytotoxicity with doxorubicin in breast cancer cells. *Tumour Biol* 35: 10201-10212, 2014.
- Fang Z, Tang Y, Jiao W, Xing Z, Guo Z, Wang W, Xu Z and Liu Z: Nitidine chloride induces apoptosis and inhibits tumor cell proliferation via suppressing ERK signaling pathway in renal cancer. *Food Chem Toxicol* 66: 210-216, 2014.
- Fang Z, Tang Y, Jiao W, Xing Z, Guo Z, Wang W, Shi B, Xu Z and Liu Z: Nitidine chloride inhibits renal cancer cell metastasis via suppressing AKT signaling pathway. *Food Chem Toxicol* 60: 246-251, 2013.
- Kang M, Ou H, Wang R, Liu W and Tang A: The effect of nitidine chloride on the proliferation and apoptosis of nasopharyngeal carcinoma cells. *J BUON* 19: 130-136, 2014.
- Liu N, Li P, Zang S, Liu Q, Ma D, Sun X and Ji C: Novel agent nitidine chloride induces erythroid differentiation and apoptosis in CML cells through c-Myc-miRNAs axis. *PLoS One* 10: e0116880, 2015.
- Ottaviani G and Jaffe N: The epidemiology of osteosarcoma. *Cancer Treat Res* 152: 3-13, 2009.
- Mirabello L, Troisi RJ and Savage SA: Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the Surveillance, Epidemiology, and End Results Program. *Cancer* 115: 1531-1543, 2009.
- Huh WW, Holsinger FC, Levy A, Palla FS and Anderson PM: Osteosarcoma of the jaw in children and young adults. *Head Neck* 34: 981-984, 2012.
- Gaffney R, Unni KK, Sim FH, Slezak JM, Esther RJ and Bolander ME: Follow-up study of long-term survivors of osteosarcoma in the prechemotherapy era. *Hum Pathol* 37: 1009-1014, 2006.
- Mohseny AB, Machado I, Cai Y, Schaefer KL, Serra M, Hogendoorn PC, Llombart-Bosch A and Cleton-Jansen AM: Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. *Lab Invest* 91: 1195-1205, 2011.
- Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB and Ivaska J: Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Ax1 expression in breast cancer. *Oncogene* 30: 1436-1448, 2011.
- Rosanò L, Cianfrocca R, Spinella F, Di Castro V, Nicotra MR, Lucidi A, Ferrandina G, Natali PG and Bagnato A: Acquisition of chemoresistance and EMT phenotype is linked with activation of the endothelin A receptor pathway in ovarian carcinoma cells. *Clin Cancer Res* 17: 2350-2360, 2011.
- Elloul S, Vaksman O, Stavnes HT, Trope CG, Davidson B and Reich R: Mesenchymal-to-epithelial transition determinants as characteristics of ovarian carcinoma effusions. *Clin Exp Metastasis* 27: 161-172, 2010.
- Liu J, Gao L, Zhang H, Wang D, Wang M, Zhu J, Pang C and Wang C: Succinate dehydrogenase 5 (SDH5) regulates glycogen synthase kinase 3 β -catenin-mediated lung cancer metastasis. *J Biol Chem* 288: 29965-29973, 2013.
- Zhou SL, Zhou ZJ, Hu ZQ, Li X, Huang XW, Wang Z, Fan J, Dai Z and Zhou J: CXCR2/CXCL5 axis contributes to epithelial-mesenchymal transition of HCC cells through activating PI3K/Akt/GSK-3 β /Snail signaling. *Cancer Lett* 358: 124-135, 2015.
- Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A and Kaibuchi K: GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 120: 137-149, 2005.
- Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X and He X: Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108: 837-847, 2002.
- Zhang X, Chen T, Zhang J, Mao Q, Li S, Xiong W, Qiu Y, Xie Q and Ge J: Notch1 promotes glioma cell migration and invasion by stimulating β -catenin and NF- κ B signaling via AKT activation. *Cancer Sci* 103: 181-190, 2012.
- Yuan H, Kajiyama H, Ito S, Yoshikawa N, Hyodo T, Asano E, Hasegawa H, Maeda M, Shibata K, Hamaguchi M, *et al*: ALX1 induces snail expression to promote epithelial-to-mesenchymal transition and invasion of ovarian cancer cells. *Cancer Res* 73: 1581-1590, 2013.
- Mitra A, Mishra L and Li S: EMT, CTCs and CSCs in tumor relapse and drug-resistance. *Oncotarget* 6: 10697-10711, 2015.
- Qureshi R, Arora H and Rizvi MA: EMT in cervical cancer: Its role in tumour progression and response to therapy. *Cancer Lett* 356: 321-331, 2015.
- Somanath PR, Vijai J, Kichina JV, Byzova T and Kandel ES: The role of PAK-1 in activation of MAP kinase cascade and oncogenic transformation by Akt. *Oncogene* 28: 2365-2369, 2009.
- de Araújo WM, Vidal FC, de Souza WF, de Freitas JC Jr, de Souza W and Morgado-Diaz JA: PI3K/Akt and GSK-3 β prevents in a differential fashion the malignant phenotype of colorectal cancer cells. *J Cancer Res Clin Oncol* 136: 1773-1782, 2010.

30. Zhang B, Yang Y, Shi X, Liao W, Chen M, Cheng AS, Yan H, Fang C, Zhang S, Xu G, *et al*: Proton pump inhibitor pantoprazole abrogates adriamycin-resistant gastric cancer cell invasiveness via suppression of Akt/GSK- β / β -catenin signaling and epithelial-mesenchymal transition. *Cancer Lett* 356: 704-712, 2015.
31. Chen J, Chan AW, To KF, Chen W, Zhang Z, Ren J, Song C, Cheung YS, Lai PB, Cheng SH, *et al*: SIRT2 overexpression in hepatocellular carcinoma mediates epithelial to mesenchymal transition by protein kinase B/glycogen synthase kinase-3 β / β -catenin signaling. *Hepatology* 57: 2287-2298, 2013.
32. Liu ZC, Wang HS, Zhang G, Liu H, Chen XH, Zhang F, Chen DY, Cai SH and Du J: AKT/GSK-3 β regulates stability and transcription of snail which is crucial for bFGF-induced epithelial-mesenchymal transition of prostate cancer cells. *Biochim Biophys Acta* 1840: 3096-3105, 2014.
33. Liu CW, Li CH, Peng YJ, Cheng YW, Chen HW, Liao PL, Kang JJ and Yeng MH: Snail regulates Nanog status during the epithelial-mesenchymal transition via the Smad1/Akt/GSK3 β signaling pathway in non-small-cell lung cancer. *Oncotarget* 5: 3880-3894, 2014.
34. Lu ZY, Dong R, Li D, Li WB, Xu FQ, Geng Y and Zhang YS: SNAIL overexpression induces stemness and promotes ovarian cancer cell invasion and metastasis. *Oncol Rep* 27: 1587-1591, 2012.
35. Surh YJ: Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 3: 768-780, 2003.
36. Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP and Gescher AJ: Dietary polyphenolic phytochemicals - promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int J Cancer* 120: 451-458, 2007.
37. Weng CJ and Yen GC: Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: Phenolic acids, monophenol, polyphenol, and their derivatives. *Cancer Treat Rev* 38: 76-87, 2012.
38. Turley EA, Veiseh M, Radisky DC and Bissell MJ: Mechanisms of disease: Epithelial-mesenchymal transition - does cellular plasticity fuel neoplastic progression? *Nat Clin Pract Oncol* 5: 280-290, 2008.
39. Hou CH, Lin FL, Hou SM and Liu JF: Cyr61 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by Raf-1/MEK/ERK/Elk-1/TWIST-1 signaling pathway. *Mol Cancer* 13: 236, 2014.
40. Guo X, Zhang J, Pang J, He S, Li G, Chong Y, Li C, Jiao Z, Zhang S and Shao M: MicroRNA-503 represses epithelial-mesenchymal transition and inhibits metastasis of osteosarcoma by targeting c-myc. *Tumour Biol*: Jan 14, 2016 (Epub ahead of print).
41. Zhang X, Jiang D, Jiang W, Zhao M and Gan J: Role of TLR4-mediated PI3K/AKT/GSK-3 β signaling pathway in apoptosis of rat hepatocytes. *Biomed Res Int* 2015: 631326, 2015.
42. Lin L, Chen YS, Yao YD, Chen JQ, Chen JN, Huang SY, Zeng YJ, Yao HR, Zeng SH, Fu YS, *et al*: CCL18 from tumor-associated macrophages promotes angiogenesis in breast cancer. *Oncotarget* 6: 34758-34773, 2015.
43. Liao K, Li J and Wang Z: Dihydroartemisinin inhibits cell proliferation via AKT/GSK3 β /cyclinD1 pathway and induces apoptosis in A549 lung cancer cells. *Int J Clin Exp Pathol* 7: 8684-8691, 2014.
44. Doble BW and Woodgett JR: GSK-3: Tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116: 1175-1186, 2003.
45. Ngo HK, Lee HG, Piao JY, Zhong X, Lee HN, Han HJ, Kim W, Kim DH, Cha YN, Na HK, *et al*: *Helicobacter pylori* induces Snail expression through ROS-mediated activation of Erk and inactivation of GSK-3 β in human gastric cancer cells. *Mol Carcinog*: Jan 25, 2016 (Epub ahead of print). doi: 10.1002/mc.22464.
46. Zhang B, Yin C, Li H, Shi L, Liu N, Sun Y, Lu S, Liu Y, Sun L, Li X, *et al*: Nrl1 promotes invasion of breast cancer cells by binding to chemokine (C-C motif) ligand 18 through the PI3K/Akt/GSK3 β /Snail signalling pathway. *Eur J Cancer* 49: 3900-3913, 2013.
47. Zhang J, Wei J, Lu J, Tong Z, Liao B, Yu B, Zheng F, Huang X, Chen Z, Fang Y, *et al*: Overexpression of Rab25 contributes to metastasis of bladder cancer through induction of epithelial-mesenchymal transition and activation of Akt/GSK-3 β /Snail signaling. *Carcinogenesis* 34: 2401-2408, 2013.
48. Wang H, Wang HS, Zhou BH, Li CL, Zhang F, Wang XF, Zhang G, Bu XZ, Cai SH and Du J: Epithelial-mesenchymal transition (EMT) induced by TNF- α requires AKT/GSK-3 β -mediated stabilization of snail in colorectal cancer. *PLoS One* 8: e56664, 2013.
49. Maseki S, Ijichi K, Tanaka H, Fujii M, Hasegawa Y, Ogawa T, Murakami S, Kondo E and Nakanishi H: Acquisition of EMT phenotype in the gefitinib-resistant cells of a head and neck squamous cell carcinoma cell line through Akt/GSK-3 β /snail signalling pathway. *Br J Cancer* 106: 1196-1204, 2012.
50. Cano A, Pérez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F and Nieto MA: The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2: 76-83, 2000.