Nitidine chloride inhibits renal cancer cell metastasis via suppressing AKT signaling pathway

Zhiqing Fang a,b, Yueqing Tang a, Wei Jiao a, Zhaoquan Xing a, Zhaoxin Guo a, Weichang Wang a, Benkang Shi a, Zhonghua Xu a, Zhaoxu Liu a,c,⇑

a Department of Urology, Qilu Hospital of Shandong University, J'nan, Shandong, China
b The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Public Health, Qilu Hospital of Shandong University, J'nan, Shandong, China
c School of Nursing, Shandong University, J'nan, Shandong, China

ABSTRACT

Nitidine Chloride (NC) has been shown to have anti-cancer effects on various tumors. However, whether NC could exert anti-metastasis activity in renal cancer cells and the underlying mechanisms have not been elucidated. In this work, our data demonstrated the anti-metastasis effects of NC on renal cancer cells in vitro. With scratch assay and transwell assays, we found that NC potently suppressed the migration and invasion of 786-O and A498 cells. Mechanistically, we presented that NC significantly decreased phosphorylation of AKT, accompanied by down-regulation of MMP-2 and MMP-9. Furthermore, a specific AKT inhibitor, LY294002, could enhance the anti-metastasis effects of NC, which indicated that NC suppressed metastasis of renal cancer cells partly via inhibition of AKT activity. Taken together, our results imply that NC can be developed as a potential anti-metastasis agent to renal cancer.

1. Introduction

Renal cell carcinoma (RCC) accounts for roughly 2–3% of all adult cancers (Baaten et al., 2004; Gupta et al., 2008; Schrader and Hofmann, 2008). Surgical intervention is the primary treatment for localized RCC. However, 30% of patients undergoing nephrectomy for localized disease eventually develop metastases (Rabinovitch et al., 1994; Lam et al., 2005). The clinical course of this disease can vary, and only 50% of patients with metastatic RCC (mRCC) are living 1 year after diagnosis, and about 10% of patients may survive longer than 5 years (Vogelzang and Stadler, 1998; Laird et al., 2013). mRCC is largely refractory to chemotherapy, radiotherapy and hormonal therapy (Wu et al., 2012). New treatments are needed to develop strategies for controlling mRCC and improving quality of life.

Nitidine is derived from the root of Zanthoxylum nitidum. Previous studies reported that Nitidine has anti-inflammatory, anti-fungal, anti-oxidant and even anti-HIV functions (Del Poeta et al., 1999; Hu et al., 2006). Nitidine Chloride (NC, Fig. 1A), a chloride of Nitidine, has been identified as a potential anti-tumor drug because it can inhibit proliferation and induce cell apoptosis in several tumor types including osteosarcoma, gastric cancer and liver cancer. (Lv et al., 2011; Chen et al., 2012; Liao et al., 2013). Meanwhile, NC has shown its anti-metastasis activity in breast cancer cells by suppressing c-Src/FAK associated signaling pathway (Pan et al., 2011). However, the function of anti-metastasis activity in renal cancer cells and the underlying molecular mechanisms have not been well established.

In this study, our data established the migration and invasion inhibitory effects of NC on renal cancer cells in vitro. We also discovered that NC could inhibit activation of AKT in renal cancer cells. Furthermore, we elucidated that NC could downregulate MMPs through inhibiting AKT kinase in renal cancer cells. Taken together, these novel findings contribute to illuminating the molecular mechanisms underlying the anti-metastasis effect of NC and identifying AKT pathway as a promising target for exploiting NC as chemotherapy drugs.
2. Materials and methods

2.1. Cell lines and reagents

Human 786-O and A498 cell lines were obtained from American Type Culture Collection (ATCC) and cultured in DMEM high Glucose (HyClone) with 10% Fetal Bovine Serum (FBS) under the conditions of 5% CO\textsubscript{2} at 37\degree C. Nitidine Chloride was purchased from Tauto Biotech (Shanghai, China) and prepared in dimethyl sulfoxide (DMSO). Antibodies against phospho-AKT, AKT, GAPDH and HRP-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against MMP-2 and MMP-9 were obtained from Immuno-Way (Newark, DE, USA).

2.2. In vitro scratch assay

Cell migration was assessed by scratch assay. The 786-O and A498 cells were seeded in 24-well plates. After incubation with NC (5 \mu M) for 24 h, each well was manually scratched with a 200 \mu l pipette tip, and then incubated at 37\degree C with NC (5 \mu M). The scratch area was photographed after 24 h. The distance between two cell edges were analyzed by ImageJ software.

2.3. In vitro invasion assay

The transwell system (24 wells, 8 \mu m pore size with poly-carbonate membrane; Corning Costar, Lowell, MA, USA) coated with 2 mg/ml basement membrane Matrigel (BD Biosciences) was used for the in vitro invasion assays. 786-O and A498 cells were seeded in 24-well plates. After incubation with NC (5 \mu M) for 24 h, each well was manually scratched with a 200 \mu l pipette tip, and then incubated at 37\degree C with NC (5 \mu M). The scratch area was photographed after 24 h. The distance between two cell edges were analyzed by ImageJ software.

2.4. In vitro migration assay

For migration assay, the cells were seeded in upper chambers without coated Matrigel. The rest of assay was performed as the invasion assay. The cells on lower surface were also counted in five randomly fields, then the cell number was analyzed statistically.

2.5. Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect cell viability. The 786-O (2 \times 10^3 cells/well) and A498 cells (3 \times 10^3 cells/well) in 100 \mu l medium were seeded in 96-well plates. After 12 h the medium in each well was replaced with the medium containing different concentration of NC and incubated for 24 h. Then, test cells were incubated with 20 \mu l MTT for 4 h at 37\degree C, the supernatant was removed and 200 \mu l of DMSO was added to each well. The absorbance values were determined by the Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.6. Apoptosis assay

Apoptosis assay was performed by flow cytometry after staining with FITC-conjugated annexin V and propidium iodide (PI), as described previously (Fang et al., 2013). In brief, harvested cells were resuspended in 100 \mu l of the binding buffer. Then, 5 \mu l of annexin V-FITC and 5 \mu l of PI (20 \mu g/ml) were added and incubated for 15 min. Finally, binding buffer (400 \mu l) was added to each reaction tube and the cells were analyzed by flow cytometry. The data was analyzed by WinMDI V2.9 software (The Scripps Research Institute, San Diego, CA, USA).

2.7. Western blot analysis

Cells were washed with PBS and lysed in RIPA buffer in the presence of protease inhibitors. Protein (50 \mu g) was separated by SDS–PAGE and transferred onto a PVDF membrane (Millipore, Bedford, Massachusetts, USA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk at room temperature, the membrane was incubated overnight at 4\degree C with the primary anti-
bodies. After washing the membrane in PBST three times, secondary antibodies conjugated with HRP were applied for 2 h at room temperature. Protein bands were visualized by enhanced chemiluminescence (Millipore). Protein levels were detected using chemiluminescence reader ImageQuant LAS4000 (GE, USA).

2.8. Statistical analysis

The student’s two-tailed t-test was used to determine statistical differences between treatment and control values. Results were considered statistically significant only if the p value was less than 0.05. All data are presented as the mean ± SD of three independent experiments.

3. Result

3.1. NC inhibits migration and invasion of renal cancer cells

The scratch assay was implemented to investigate the effect of NC on the migration of renal cancer. As shown in Fig. 1B and C, migration of 786-O cells was restrained by NC (5 μM). The similar anti-migration effect was also observed in A498 cells (Fig. 1D and E).

To further test the influence of NC on cell migration and invasion, 786-O and A498 cells treated with NC (5 μM) were used to conduct the transwell assay. Our result testified that NC could significantly inhibit the migration and invasion of 786-O cell (**P < 0.01) (Fig. 2A and B). Furthermore, we observed the same potent effect of NC on A498 cells (Fig. 2C and D), indicating that NC could inhibit the migratory and invasive ability of renal cancer cells (**P < 0.01).

3.2. The anti-metastasis effect of NC in renal cancer cells is partially due to its inhibitory effect on proliferation

To study whether the anti-metastasis effect of NC was due to the inhibitory effect of NC on proliferation in RCC cells, 786-O and A498 cells were exposed to different concentrations of NC for MTT assay. We noticed that NC could significantly inhibit the proliferation of 786-O cells at the concentration of 5 μM. However, the 5 μM of NC did not cause significant inhibition on cell proliferation in A498 cells (Fig. 3A and B). To further detect and quantify the apoptotic cells induced by NC (5 μM), we used annexin V-conjugated FITC and propidium iodide staining to analyze the percentage of apoptotic cells. The total percentage of apoptotic cells (UR + LR) increased from 2.08% in non-NC treated 786-O cells to 16.07% in NC-treated (5 μM) cells (Fig. 3C), while the total percentage of apoptotic cells was increased from 2.85% to 9.46% in A498 cells (Fig 3D). We also adopted flow cytometry to test the cell cycle distribution of RCC cells after treatment with NC; however, we found no significant effect of NC (5 μM) on cell cycle distribution in RCC cells (data not shown).

Although the apoptotic rate was 16.07% for 786-O cells treated with NC (5 μM), the migration and invasion inhibitory rate was over 50%. Additionally, the concentration (5 μM) adopted for scratch and transwell assay did not cause significant inhibition on cell proliferation in A498 cells, indicating the anti-metastasis effect of NC was partially due to its inhibitory effect on proliferation. Thus, we speculated other mechanisms may account for the anti-metastasis effect of NC in renal cancer cells.

Fig. 2. Cell migration and invasion ability were inhibited by NC via transwell assay. (A) Migration and invasion of 786-O cells was inhibited after treatment NC (5 μM). (B) The number of 786-O cells that successfully migrated and invaded was counted. (C) Migration and invasion of A498 cells was inhibited after treatment NC (5 μM). (D) The decreased number of A498 cells indicated the great inhibitory effect of NC on cell mobility. Data is presented as mean ± SD of three independent experiments. **P < 0.01.
3.3. NC-induced suppressive effect on MMP-2/9 expression is accompanied by down-regulation of AKT signaling

Recent data provide clear evidence that AKT signaling, constitutively activated in various human cancers, including prostate cancer, breast cancer as well as renal cancer, plays a critical role in cancer progression (Blume-Jensen and Hunter, 2001; Brugge et al., 2007; Pal and Mandal, 2012). Therefore, we speculate whether AKT signaling plays a role in NC-induced metastatic inhibition. As shown in Fig. 4A, after treated with NC (5 and 10 μM) for 24 h, the phosphorylation level of AKT was effectively suppressed in both 786-O and A498 cells. Correspondingly, the expression of MMP-9 decreased significantly after NC treatment in both cell lines, and the expression of MMP-2 was also suppressed in 786-O cells. However, NC weakly inhibited the activity of MMP-2 in A498 cells.

3.4. Inhibition of AKT signaling enhanced the anti-metastasis effect of NC in renal cancer cells

LY294002, a pan-PI3K inhibitor, could potently inhibit AKT activation. To examine whether blockade of AKT signaling with LY294002 could potentiate the ability of NC to inhibit cell proliferation, migration and invasion of renal cancer cell, 786-O and A498 cells were cultured in the presence of either NC (5 μM) or/and LY294002 (50 μM) for 24 h. The protein level of p-AKT, AKT, MMP-2/9 were detected via western blotting. In Fig. 4B, the results demonstrated that either LY294002 or NC could significantly suppress the AKT activation and MMP-2/9 expression. Nevertheless, the combined treatment with LY294002 could enhance the inhibitory effect of NC on AKT activation and MMP-2/9 expression, suggesting that NC exerted its functions in renal cancer cells partly via inhibition of AKT activity.

Next, we examined whether the anti-metastasis effect of NC was attributed to AKT signaling suppression. We first tested the effect of LY294002 on cell proliferation in the presence or absence of NC.

Fig. 3. The anti-metastasis effect of NC in renal cancer cells is partially due to its inhibitory effect on proliferation. (A) 786-O cells and (B) A498 cells were treated with NC for 24 h. NC inhibited cell viability of both cell lines in a dose-dependent manner. The results represent as the mean ± SD of three independent experiments and the corresponding standard error. *P < 0.05; **P < 0.01. (C) 786-O and (D) A498 cells were treated with NC (3 μM) for 24 h and stained with annexin V-FITC and PI. The percentage of early stage of apoptosis and late stage of apoptosis were shown in the upper right and lower right quadrants, respectively.

Fig. 4. NC inhibited p-AKT, MMP-2 and -9 expressions. (A) Representative Western blot showing changes in the protein levels of AKT, MMP-2 and MMP-9 in renal cancer cells after exposure to NC. The 786-O and A498 were treated with NC (5 and 10 μM) for 48 h, and then the total protein was prepared and determined as described in materials and methods. The levels of AKT, p-AKT and MMP-2 and -9 expressions were estimated by western blotting analysis with GAPDH as a control. (B) After 786-O and A498 cells were treated with NC (5 μM) and/or LY294002 (50 μM) for 24 h, the levels of AKT, p-AKT and MMP-2 and -9 were analyzed by western blotting with GAPDH as a control.
NC. As shown in Fig. 5A and B, LY294002 could potentiate the anti-proliferation effects of NC, although treatment with LY294002 alone resulted in moderate anti-proliferative effects. However, we observed more significant inhibitory effect on metastasis in these two cell lines (Fig. 5C and D), reinforce the idea that inhibition of metastasis by NC is mediated by down-regulation of MMP-2/9 though Akt signaling in renal cancer cells.

4. Discussion

Accumulating evidence has underscored the anti-cancer efficacy of NC against multiple malignant tumors (Pan et al., 2011; Chen et al., 2012; Liao et al., 2013). However, whether NC could inhibit renal cancer cell migration and invasion in vitro have not been elucidated. In this study, we demonstrated for the first time that NC could effectively inhibit migration and invasion of renal cancer cells in vitro. We further explored that AKT activation was required in NC-induced anti-metastasis effect in renal cancer cells. Our data provided a new mechanism by which NC exhibits its anti-metastasis effect in renal cancer cells.

The Akt signaling pathway plays critical roles in regulating cell viability and motility in a variety of stimuli (Qian et al., 2009; Srinivasan et al., 2009). It has also been reported that the expression of phosphorylated Akt in neoplastic renal tissue was significantly greater than that in the adjacent non-neoplastic renal tissue and elevated Akt activation was often found in high-grade and mRCC (Horiguchi et al., 2003; Kuroda et al., 2009). Nowadays, the inhibition of Akt phosphorylation has been suggested as a novel target for therapeutic agents in a large scale of human cancers (Fresno Vara et al., 2004). In our study, we have shown that NC could downregulate the Akt activity, and inhibited Akt activity by LY294002 could markedly increase NC-induced anti-metastasis effect. These results suggest that NC-induced anti-cancer effect is associated with Akt inactivation.

The process of invasion and metastasis of renal cancer is complex and multiple factors may profoundly be involved in the process. MMPs are shown to be crucial proteinases in tumor growth as well as in the multistep processes of invasion and metastasis (Himelstein et al., 1998; Kallakury et al., 2001; Hu and Ivashkiv, 2006), including proteolytic degradation of extracellular matrix, alteration of the cell–cell extracellular matrix interactions, migration and angiogenesis (Gialeti et al., 2011). Increased expression of MMP-2 and MMP-9 proteins in renal cell carcinoma patients correlate with poor prognostic variables including shortened patient survival (Kallakury et al., 2001).

We assessed the inhibitory effect of NC on the migration and invasion of 786-O and A498 cells by means of scratch and transwell assays. Further, we detected the protein level of MMP-2 and MMP-9 in 786-O and A498 cells after treated with NC and found that decreased expression of MMP2 and MMP9 after NC treatment was related to the inhibition of migration and invasion in 786-O and A498 cells.

Fig. 5. Inhibition of AKT signaling enhanced the anti-proliferation and anti-metastasis effect of NC in renal cancer cells. After treated with NC (5 μM) and/or LY294002 (50 μM) for 24 h, (A) 786-O and (B) A498 cell proliferation was determined by MTT assay. (C) 786-O and (D) A498 cell migration was measured by transwell assay. The results represent as the mean ± SD of three independent experiments. *P < 0.05 or **P < 0.01, compared with the cells treated with DMSO (Control). #P < 0.05 or ##P < 0.01, compared with the cells treated with NC.
Fig. 6. A schematic model: the mechanisms through which NC suppresses the metastasis of renal cancer cells.

Besides participating in the regulation of cancer cell survival, Akt can regulate the expressions of several metastasis-related proteins include MMPs (Kosmidou et al., 2001; Ahmad et al., 2012; Tong et al., 2013; Zhou et al., 2013). Our data also suggested there was a relationship between the decreased activity of AKT and decreased expression of MMP-2 and MMP-9 in NC-treated renal cancer cells. Inhibition of AKT activity could enhance the anti-metastasis effect of NC in renal cancer cells, which suggested that AKT may be the upstream mediator of NC-induced anti-metastasis in renal cancer cells (Fig. 6).

In summary, our results imply that AKT signaling pathway plays a key role in NC induced anti-metastasis effect in renal cancer cells. All these results suggest a protective activity of NC in renal cell carcinoma development and metastasis.

Conflict of Interest

All authors declare no conflict of interest.

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References


