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Nitidine chloride efficiently induces autophagy and apoptosis in melanoma cells via AMPK-mTOR signaling pathway

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Nitidine chloride (NC) has displayed anti-tumor effects in various types of cancer. However, the role of NC in human melanoma is largely unknown. This study aimed to investigate the effects of NC on melanoma cancer cells A375 and WM35 by MTT assay. Apoptosis was measured by detecting caspase-3 protein expression level and its activity. Autophagy was measured by TEM image, immunostaining and immunoblotting assays. MTT assays showed that NC significantly blocks melanoma cells proliferation. Immunoblotting and caspase-3 activity assays showed that NC inhibited melanoma cells proliferation by inducing cell apoptosis. TEM, immunostaining and immunoblotting assays showed that NC also triggers melanoma cells autophagy and activation of the AMPK-mTOR pathway, which plays an important role in autophagy initiation. Finally, we found that blocking autophagy by 3-MA or AMPK pathway inhibitor greatly enhanced NC-induced apoptosis and cell death, indicating that NC-induced autophagy may have a cytoprotective effect in melanoma cells. Together, these results suggested that NC has strong anti-tumor effects on melanoma cells.

1. Introduction

Melanoma, arising from pigment-producing melanocytes, is the most aggressive malignant skin cancer and accounts for approximately 80% of all skin cancer related deaths (Villanueva et al. 2010). Although significant advances have been made, the effect of chemotherapy treatment of melanoma is still limited, with a five-year survival rate below 15% (Cummins et al. 2006; Miller and Mihm 2006). Thus, it remains urgent to develop novel anti-melanoma cancer agents.

Nitidine chloride (NC) is a phytochemical alkaloid isolated from the roots of *Zanthoxylum nitidum* (Roxb) (Liu et al. 2014). Previous studies have demonstrated that NC has strong anti-tumor effects in various types of cancer, such as renal cancer (Cournoyer et al. 2012), breast cancer, hepatocellular carcinoma cells, colorectal cancer cells and nasopharyngeal carcinoma cells (Kang et al. 2014; Ou et al. 2015; Sun et al. 2016a; Zhai et al. 2016). However, whether NC has anti-tumor effects on melanoma cells has not been elucidated.

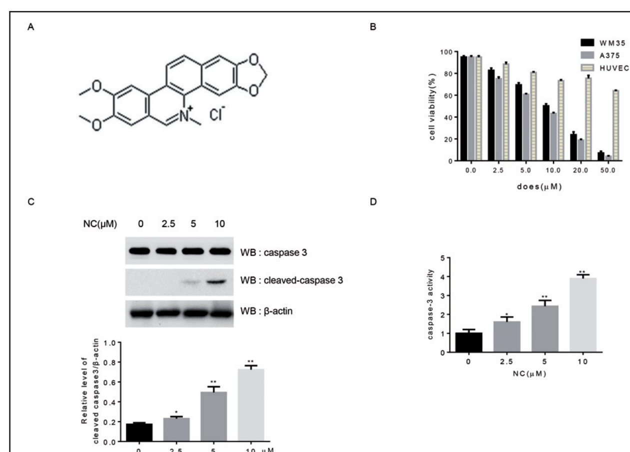
Autophagy is a process that facilitates the cellular integrity and energy homeostasis by degrading the components in autophagosomes, which involves dysfunctional organelles, misfolded proteins and other cytoplasmic components. Many natural products can induce both apoptosis and autophagy. Recent studies showed that autophagy and apoptosis share similar effectors and could be induced by the same stimuli. However, the relationship between autophagy and apoptosis is complicated (Mukhopadhyay et al. 2014). Therefore, whether NC could induce autophagy in melanoma cells and the relationship between NC-induced autophagy and apoptosis needs to be elucidated.

In this study, we demonstrated that NC has anti-tumor effects on human melanoma cell lines A375 and WM35 by inducing caspase-dependent apoptosis. NC could also induce protective autophagy through the AMPK-mTOR pathway. Moreover, autophagy inhibition by 3-MA or AMPK pathway inhibitor greatly increases NC-induced apoptosis. These results suggest that NC-induced autophagy might have a cytoprotective effect in melanoma cells.

2. Investigations and results

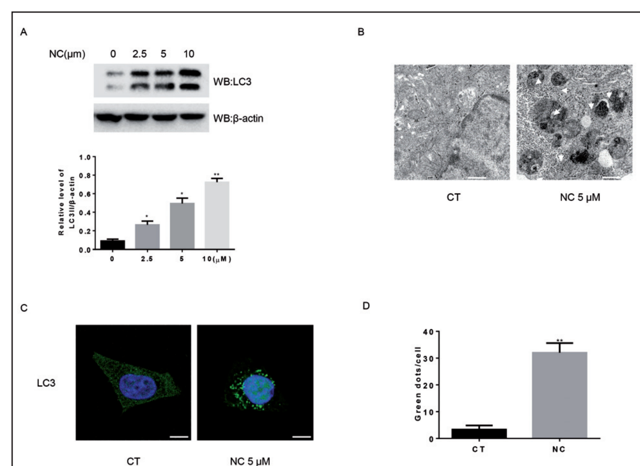
2.1. NC induces apoptotic cell death in human melanoma cells

To investigate whether NC has an anti-tumor effect on human melanoma cells, we performed MTT assays. Increased concentrations (2.5, 5, 10, 20 and 50 μM) of NC were added in melanoma cell lines (A375 and WM35) and non-malignant cell HUVECs for 48 h. The results showed that NC dramatically suppressed A375 and WM35 cell growth in a dose-dependent manner with IC50 values of 7.67 μM in A375 cells and 9.34 μM in WM35 cells, respectively .However, the non-malignant HUVEC cells were not much affected (Fig. 1b). To determine whether NC inhibited melanoma cell proliferation through apoptosis, we examined the protein level of caspase-3. After treatment with various concentrations of NC in A375 cells, we found the level of cleaved caspase-3 dramatically increased, which indicates the initiation of apoptosis (Fig. 1c). In addition, caspase-3 activity was also upregulated after NC treatment (Fig. 1d). The results indicated that NC could induce caspase-dependent apoptosis in melanoma cell lines.



2.2. *NC induces autophagy in melanoma cell lines*

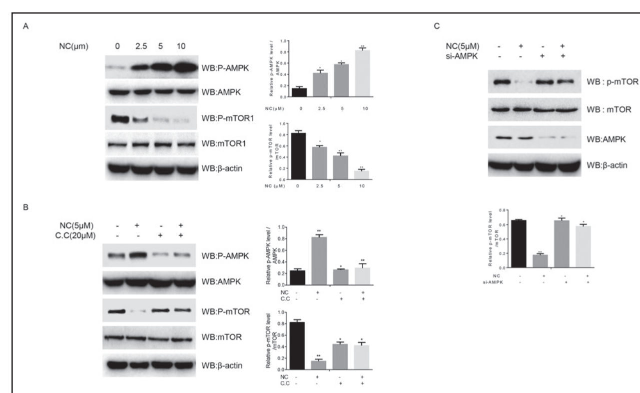
To investigate whether NC induce autophagy in A375 cells, we examined the accumulation of LC3II by immunoblotting assay. As shown in Fig. 2a, NC treatment dose-dependently increased the expression level of LC3II in A375 cells. Moreover, TEM images showed that autophagosomes were accumulated in NC-treated A375 cells as compared with the untreated group (Fig. 2b). In addition, the green puncta of LC3 was triggered after NC treatment (Fig. 2c and 2d). These results showed that NC could induce autophagy in melanoma cells.



2.3. *NC induces autophagy through upregulation of the AMPK-mTOR pathway*

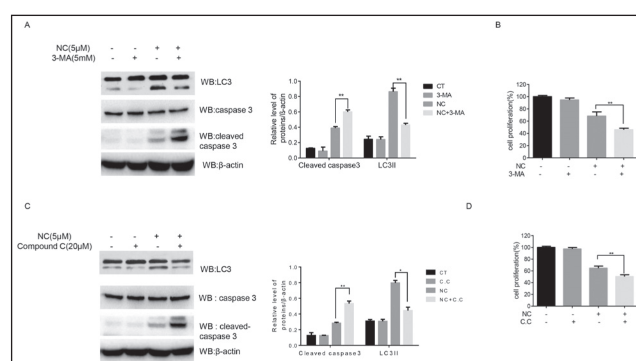
AMPK and mTOR are two key regulators in autophagy, activation of AMPK or repression of mTOR could trigger autophagy initiation (Egan et al. 2011; Inoki et al. 2003). We therefore determined whether these processes were involved in NC-induced autophagy by measuring the phosphorylation level of AMPK and mTOR in A375 cells. As shown in Fig. 3a, NC treatment significantly upregulated the phosphorylation level of AMPK but downregulated mTOR phosphorylation level, indicating that AMPK and mTOR pathways were both involved in NC-induced autophagy.

Activated AMPK could directly phosphorylate TSC2, leading to repression of the mTOR pathway (Inoki et al. 2003). To investigate whether NC inhibits mTOR phosphorylation dependent on AMPK activation, Compound C (C.C), an AMPK inhibitor was used. As shown in Fig. 3b, C.C treatment greatly inhibited AMPK activation in A375 cells. Moreover, after treatment with C.C, NC-induced inhibition of p-mTOR was significantly attenuated. We further confirmed this result using si-RNA to knockdown AMPK in A375 cells. As expected, mTOR phosphorylation was marginally decreased upon NC treatment in AMPK knockdown A375 cells as compared with control (Fig. 3c). Taken together, these results demonstrated that NC induced autophagy through the AMPK-mTOR signal pathway in A375 cells.



2.3. *Inhibition of autophagy promotes NC-induced apoptosis in A375 cells*

We next investigated the functional role of NC-induced autophagy in melanoma cells by using 3-MA, an autophagy inhibitor. As expected, 3-MA greatly suppressed NC-induced autophagy as evidenced by the decreased expression level of LC3II in A375 cells (Fig. 4a). Moreover, co-treatment with 3-MA and NC significantly enhanced the cleavage of caspase-3 as compared with NC treatment alone. In addition, MTT assay showed that the inhibition rate of cell proliferation was augment in the combination group (Fig. 4b). Similarly, C.C treatment promoted NC-induced cleavage of caspase 3 and enhanced NC-induced cell death in A375 cells (Fig. 4c and 4d). These results suggested that NC-induced autophagy exerts a cytoprotective effect against apoptosis.



3. Discussion

Nitidine chloride (NC), isolated from the root of *Zanthoxylum nitidumis,* has been reported to exhibit several functions like anti-inflammatory, anti-leukemic or even anti-HIV activity (Del Poeta et al. 1999; Hu et al. 2006). NC was also reported to exert anti-tumor effects in various types of cancers. NC suppresses the Hedgehog pathway to inhibit epithelial-mesenchymal transition in breast cancer cells (Sun et al. 2016b). NC induces apoptosis in ovarian cancer cells through activating the Fas signaling pathway or inhibiting ERK signaling pathway (Chen et al. 2018; Sun et al. 2016c). Further studies reveal that NC downregulates the protein expression of YAP, DNA topoisomerase 1 and 2A or SIN1 to induce cell apoptosis in osteosarcoma cells, liver cancer or prostate cancer (Liu et al. 2018; Shi et al. 2019; Xu et al. 2019). However, the anti-cancer activity of NC in melanoma cells has not been investigated so far. In this study, we showed that NC dramatically inhibited melanoma cell proliferation through caspase-dependent apoptosis and has a potential anti-tumor effect on human melanoma cells.

Besides apoptosis, many natural products could induce autophagy to promote or inhibit tumor cell growth. Our results for the first time show that NC could induce autophagy in melanoma cells. Both the TEM and immunostaining results showed that NC treatment greatly increased the accumulation of LC3-II as compared with a control group. AMPK and mTOR are central for autophagy initiation. Activation of AMPK leads to the phosphorylation of its substrates like Beclin1, Vps34, ULK1 to promote autophagosomes formation (Li and Chen 2019). mTOR inhibition leads to the dissociation of mTORC1 from the ULK complex, resulting in the activation of ULK1 and initiation of autophagy (Mizushima 2010). Notably, activated AMPK could directly downregulate mTOR phosphorylation, thus triggering autophagy initiation (Villanueva-Paz et al. 2016). In this study, we found that NC could both upregulate AMPK phosphorylation and downregulate mTOR phosphorylation to induce autophagy. Moreover, blocking the AMPK signal pathway by an AMPK inhibitor or AMPK knockdown dramatically attenuated NC-induced suppression of mTOR, suggesting that NC inhibits mTOR phosphorylation in an AMPK dependent manner.

The relationship between autophagy and apoptosis is context dependent. Natural products like pogostone and aristolochic acid I could induce cytoprotective autophagy to suppress apoptosis in cancer cells; however, compounds like resveratrol could induce autophagic cell death to enhance apoptosis (Cao et al. 2017; Lang et al. 2015; Zeng et al. 2012). Our results demonstrated that NC-induced autophagy plays a cytoprotective role against apoptosis. Inhibition of autophagy by 3-MA or by AMPK inhibitor potentiated NC-induced caspase 3 activity and apoptotic cell death in A375 cells.

In conclusion, we demonstrated that NC has anti-tumor effects on human melanoma cell lines A375 and WM35 by inducing caspase-dependent apoptosis. In addition, NC induced cytoprotective autophagy through the AMPK-mTOR pathway. Inhibition of this autophagy potentiates NC induced apoptosis. Our findings suggest that NC is a potential anti-tumor agent and combined with an autophagy inhibitor may be a promising strategy for melanoma therapy.

4. Experimental

4.1. Materials and cell lines

Nitidine chloride (Fig. 1a) was purchased from Tauto Biotech (Shanghai, China). Compund C and 3-MA were obtained from Sigma-Aldrich (St Louis, MO). Human melanoma cell lines A375 and WM35 and human umbilical vein endothelial cells (HUVECs) were obtained from cell bank of Chinese Academy of Sciences (Shanghai, China). The A375 and WM35 cells were cultured in DMEM medium with 10% fetal bovine serum and 1% penicillin/streptomycin. HUVECs were cultured in Endothelial Cell Medium (ECM) with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were grown in a humidified incubator in 5% CO_2 at 37 °C. The antibodies were used as followed: caspase-3 (#9662), cleaved caspase-3 (#9661), LC3II (#3868), p-AMPK (Thr172) (#2535), AMPK (#5832), p-mTOR (#2971), mTOR (#2972) were from Cell Signaling Technology (Danvers, MA, USA). β-actin was obtained from Santa Curz Biotechnology (Santa Cruz, CA).

4.2. Cell viability assay

Briefly, cells were seeded into 96-well plates (5000 cells/well) for 24 h. Cells were then treated with increased concentrations (0, 2.5, 5, 10, 20 and 50 μM) of NC for 48 h. Then, cells were incubated with MTT reagent (20 μl, 5 mg/ml) for an additional 4 h at 37 °C. Cell viability was determined with a microplate reader at 570 nm.

4.3. Caspase activity assay

Caspase activity was measured using the Caspase-Glo™ 3 assay kit (Promega, Japan). Briefly, A375 cells were plated into 96-well plates (5×10^4 cells/well) and were treated with or without NC for 24 h. Cells were then incubated with the measuring reagent for 1 h at 37 °C. Caspase activity was determined by measuring the luminescence with a microplate reader.

4.4. Immunofluorescence staining

After treatment with NC for 24 h, human melanoma cell lines A375 were fixed with 100% MeOH and incubated with anti-LC3 antibody overnight. Cells were then incubated with FITC-conjugated secondary antibody for 2h in dark as previously described (Qian et al. 2020).

4.5. Transmission electron microscopy

After treated with NC for 24 h, human melanoma cell line A375 were harvested and fixed for 2 h. Cells were then post-fixed with osmium tetroxide and embedded in EMbed 812 (Electron Microscopy Sciences). After sectioned and stained, the samples were examined by using JEM-1200EX transmission electron microscope (JEOL, Japan).

4.6. Small interfering RNA transfection

A375 cells were transfected with si-RNA targeting the AMPKα1 and α2 (sc-45312 Santa Cruz, USA) and si-NC (sc-37007 Santa Cruz, USA) as a control by using Lipofectamine 2000 (Invitrogen, USA). After transfection for 6 h, the culture medium was replaced.

4.7. Statistical analyses

The data were presented as mean±SD and all of the experiments were repeated at least three times. The difference among treatment groups were performed by one-way ANOVA and Student's t-test. $p < 0.05$ considered as statistically significant.

Authors' contributions: SY and DF conceived and designed the experiments; DF, YY and SQ performed the experiments and contributed to molecular analysis; DF and YY analyzed the data; SY wrote the manuscript.

Conflicts of interest: The authors declare that they have no conflicts of interest related to this article.

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