JBUON 2014; 19(1): 130-136
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

The effect of nitidine chloride on the proliferation and apoptosis of nasopharyngeal carcinoma cells

Min Kang[1], Hesheng Ou[3], Rensheng Wang[1], Wenqi Liu[1], Anzhou Tang[2]

[1]Department of Radiotherapy, [2]Department of Ear-Nose-Throat, First Hospital of Guangxi Medical University; [3]College of Pharmacy, Nanning, Guangxi, P.R. China

Summary

Purpose: *This study was conducted to investigate the effect of nitidine chloride (NC) on the proliferation and apoptosis of nasopharyngeal carcinoma cell line CNE1, CNE2, TWO3, and C666-1, and to explore its antitumor mechanism.*

Methods: *NC was dissolved in IMDM medium and cultured with nasopharyngeal carcinoma cell line CNE1, CNE2, TWO3 and C666-1. Cell morphology, cell proliferation, cell apoptosis, p53 mRNA and p53 protein levels were assessed.*

Results: *After incubation with NC for 24 h, typical apoptotic morphology was observed. NC inhibited the proliferation and induced apoptosis of all 4 cell lines in a time-dose dependent manner. p53 mRNA and p53 protein levels were significantly increased.*

Conclusions: *NC inhibited proliferation and induced apoptosis of nasopharyngeal carcinoma cells with upregulation of p53 gene.*

Key words: *apoptosis, cell proliferation, nasopharyngeal carcinoma, nitidine chloride*

Introduction

Nasopharyngeal carcinoma occurs mainly in the coastal areas in China, such as Guangdong, Guangxi, Hunan, Jiangxi, and Fujian provinces [1,2]. The main treatment of this aggressive malignancy is radiotherapy with 5-year overall survival rate of about 70%. Chemotherapy is also an important option for patients, especially for those in late disease stages. However, owing to toxicity and side effects, chemotherapy is hard to complete. Therefore, there is an obvious need to discover more effective drugs with fewer side effects for nasopharyngeal carcinoma patients.

Nitidine, a natural compound from the root of Zanthoxylum nitidum, has shown favorable effects by virtue of its antitumor, antiinflammatory, and analgesic activities [3-6]. Nitidine-mediated cell toxicity is well known and is exerted via topoisomerases I and II [7,8]. Recently, it has been reported that NC is capable of controlling cell growth by inducing apoptosis and inhibits migration and invasion in several cancer cell lines [4,5,9]. However, it is unknown whether NC has any effect in nasopharyngeal carcinoma. In the current study, we mainly focused on the effect of NC on the proliferation and apoptosis of nasopharyngeal carcinoma cells.

Methods

Cells and reagents

NC was purchased from Tauto Biotech (Shanghai, China). The nasopharyngeal carcinoma cell lines CNE1, CNE2, TWO3 and C666-1 were preserved in our lab. These cell lines were cultured [10] in incubator with 10% inactivated newborn bovine serum (15% for C666-1 cell line), 100 U/ml penicillin and 100 U/ml streptomycin in IMDM medium (Grand Island, NY, USA) at 37 °C with relative humidity 90%, 20-21% O_2 and 5% CO_2. The culture condition and medium of C666-1 cell line were similar with those of CNE1, CNE2 and TWO3, except that the concentration of inactivated newborn bovine serum was 15%. The corresponding positive control groups of certain concentrations (cyclophosphamide/CTX) and negative control groups (IMDM

Correspondence to: Hesheng Ou, PhD. College of Pharmacy, Guangxi Medical University, Nanning, 530021, P.R. China.
Tel: +86 771 5300216, Fax: +86 771 5358272, E-mail: hsou01@126.com
Received: 22/05/2013; Accepted: 30/06/2013

medium) were established. Cells were preserved in an incubator with 5% CO_2 and 20-21% O_2, and the cell growth state was observed every day under inverted microscope.

CTX was purchased from Baxter Pharmaceutical Co, Bayern, Germany. Thiazole blue and PI dye solution were purchased from Sigma Co (St.Louis, Missouri, USA). The flow cytometry FACSCaLibur was purchased from American Beckman Coulter Company (BREA, California, USA). The pathology image analyzer Leica DM-R+Q550 was purchased from Wetzlar, Germany.

MTT

The MTT assay was modified from the procedure reported previously [10]. Cells in the logarithmic phase of growth were incubated in 96-well plate, each well containing 2×10^3 cells. IMDM medium was changed after 24 h, and 200 µl of drug solution with different concentrations were added. The corresponding negative control groups (complete culture solution of 200 µl of medium) were established. The solution was incubated in 5 % CO_2 incubator for 5 days, and the IMDM medium was changed with different concentrations of NC every 2 days. Different concentrations at each time point were added in 4 wells. Four hours prior to termination, 20 µl of MTT solution were added to each well. The supernatant was discarded and 150 µl of dimethylsulfoxide was added and gently oscillated for 10 min for complete crystal dissolving. The solution was then measured for light absorption value (A value) at 580 nm wavelength by fluorescence enzyme-linked analyzer, and the inhibition rate (IR) was calculated using the formula: IR (%) = (A value of control well–A value of experimental well)/A value of control group×100%.

Flow cytometry

The nasopharyngeal carcinoma cells were cultured in IC_{50} drug concentration (CNE1, 30.02 mg/l; CNE2, 20.16 mg/l; TWO3, 20.12 mg/l; C666-1, 30.02 mg/l) for 12 h and 1 ml of cell suspension was collected with a concentration of approximately 10^6/ml cells. The solution was centrifuged (1000r· min^{-1} for 5 min) and the medium was discarded. The cells were fixed by 70% cold ethanol at 4 °C. After double staining by PI and Annexin V-FITC, cell apoptosis was detected by flow cytometry.

RT-PCR

Total RNA extraction was performed following Trizol extraction reagent instructions. RT reaction system (4 µl of 25 mmol/l $MgCl_2$,2 µl of 10×buffer, 2 µl of dNTP, 0.5 µl of RNase inhibitor, 1 µl of ALV reverse transcriptase, 1 µl of Oligo dT, and 3 µl of RNA in each system) was in the following conditions: 3 min at 65 °C, 90 min at 42 °C, 10 min at 72 °C, and preserved at -20 °C. After the RT reaction, 5 µl of each product was taken and mixed with 1.2% agarose gel for electrophoresis (50 V for 60 min) with analysis of gray scale scanning. The results were expressed as average relative unit of 3 samples p53/β-actin. Primer of p53: sense primer was 5'-CTTACC AGGGCAACTATGGCT -3', and antisense primer was 5'-CAGGCACAA ACACGA ACCTC-3' (526 bp). Primer of β-actin: sense primer was 5'-TGGAATCCTGTGGCATCC-3', and antisense primer was 5'-TCGTACTCCTGCTTGCTG-3' (283 bp).

Western blotting

After 24-h drug treatment, the solution was washed 3 times with PBS. The cells were decomposed for protein extraction and preserved at -80 °C. Protein quantification was performed by bicinchonicic acid (BCA) colorimetric assay. The samples were electrophoresis-separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation gel, and protein was transferred to PVDF membrane. Tris-buffered saline TWEEN 20 (TBST) -prepared 5% skim milk was sealed at room temperature for 1-2 h and anti-p53 monoclonal antibody (1:100) was added. The membrane was incubated at 37 °C for 1h or 4 °C overnight, and horseradish peroxidase-labeled secondary antibody 1:1000 was added after the membrane was washed for 1 h at 37 °C in incubation. After membrane washing, luminous agent was added and the film was fixed and washed. Gray scale scanning was performed by gel imaging analysis system, and the results were expressed as p53/β-actin gray scale ratio.

Statistics

Data were expressed as mean±standard error of the mean (SEM), being the results of 3 independent experiments. Independent Student's t-test was used for comparisons between groups. One Way ANOVA test was used for multigroup comparisons. The IC_{50} of drugs on cells was determined based on the linear regression equation of drug concentration and cell survival rate. For all statistical analyses the SPSC v. 13.0 (Chicago, Illinois, USA) was used.

Results

The effect of NC on nasopharyngeal carcinoma cell growth and cell morphology

To investigate the anti-cancer effect of NC, changes of cell growth were analyzed by the MTT assay and cellular morphology was observed under inverted microscope. In the control group, cells were adherent and grew like epithelial cells; they were malleable, flat, homogeneous and transparent with clear nuclear membrane and nucleoli. Cell structure was tight, and cell growth was exuberant. On the contrary, the cell structure of the NC group and positive control group had intense outline and cells were round and float. The cell

contact was loose with slow multiplication and increased cytoplasmic particles, showing typical apoptotic morphological changes.

As shown in Figure 1, cells were cultured in drug solution with different concentrations for 5 days. The cell growth of CNE1, CNE2, TWO3 and C666-1 was inhibited in NC group in a time- and dose-dependent manner with good volume-effect and time-effect relationship. In 5 days, as the drug concentration increased, the cell inhibition rate on CNE1 increased. With the passing of time, the inhibition rate increased. After 48 h IC_{50} was 30.02 mg/l. As drug concentration increased, the inhibition rate also increased in CNE2 cells. The changes in inhibition rates caused by different drug treatment times were not as obvious as those caused by different drug concentrations. In the 48 h time period, IC_{50} was 20.16 mg/l. As drug concentration increased, the inhibition rate increased in TWO3 cells as well. The high concentration group with 40 mg/l of NC showed strong inhibition, but the inhibition rate did not increase with time. The low concentration group of 5 mg/l showed no inhibition on nasopharyngeal carcinoma cell growth. In the 48 h time period, IC_{50} was 20.12 mg/l. As drug concentration increased, the inhibition rate of C666-1 cell line also increased. With the passing of time, the inhibition rate increased. However, there was no inhibition effect of low concentration (5 mg/l). In the 48 h time period IC_{50} was 30.02 mg/l.

NC induction of apoptosis of nasopharyngeal carcinoma cells

To study further the mechanism of the NC in regulating cell growth, flow cytometry was used to measure cell apoptosis. As shown in Table 1 and Figure 2, after treatment with NC for 12 h, the apoptotic rates of CNE1, CNE2, TWO3 and C666-1 cell lines were 71.32, 70.84, 74.73 and 70.01%, respectively. Compared with the control group, this result was statistically significant ($p<0.01$), indicating that NC provoked strong apoptosis on nasopharyngeal carcinoma cell lines.

Increased induction of p53 expression in nasopharyngeal carcinoma cells

Because the results of cell growth inhibition and apoptosis of the four cancer cell lines were similar, CNE1 cell line was chosen as representative line, for studying and analyzing the NC ef-

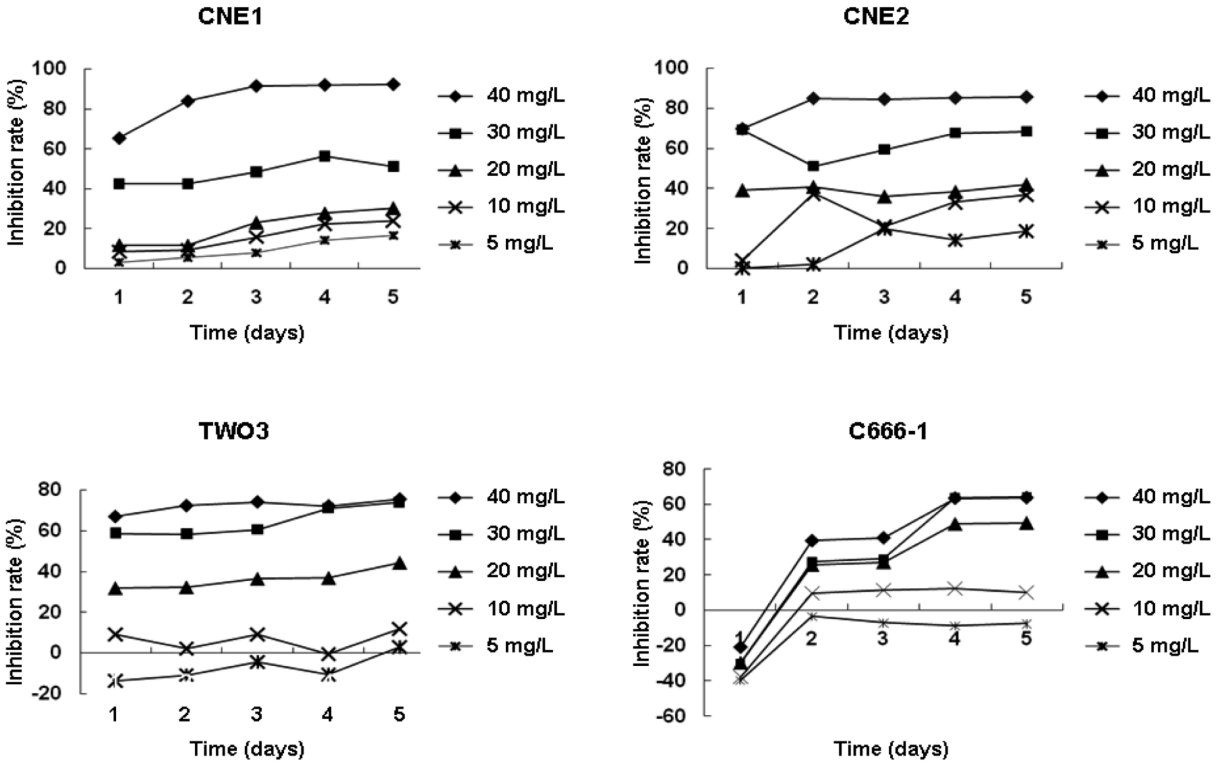

Figure 1. Growth curves of different concentrations of nitidine chloride of 4 nasopharyngeal carcinoma cell lines. Four cell lines (CNE1, CNE2, TWO3, and C666-1) were treated with nitidine chloride at different concentrations for 5 days. Cell growth was measured by MTT assay. The IC_{50} in each group was measured at 48 h post-treatment. The results show that the growth of CNE1, CNE2, TWO3 and C666-1 was inhibited by nitidine chloride treatment in a time- and dose-dependent manner.

Table 1. The apoptostic effect of NC on 4 nasopharyngeal carcinoma cell lines ($\bar{x}$ ± S, n = 3).

Group	*CNE1 (%)*	*CNE2 (%)*	*TWO3 (%)*	*C666-1 (%)*
Control group	7.13 ± 1.38	8.64 ± 1.32	7.82 ± 2.64	6.34 ± 2.57
NC group	71.32 ± 3.42*	70.84 ± 3.39*	74.73 ± 5.32*	70.01 ± 3.43*
CTX group	85.35 ± 2.44*	84.44 ± 4.73*	84.75 ± 5.83*	81.94 ± 6.34*

*p < 0.01, compared with control group. NC: nitidine chloride, CTX: cyclophosphasmide

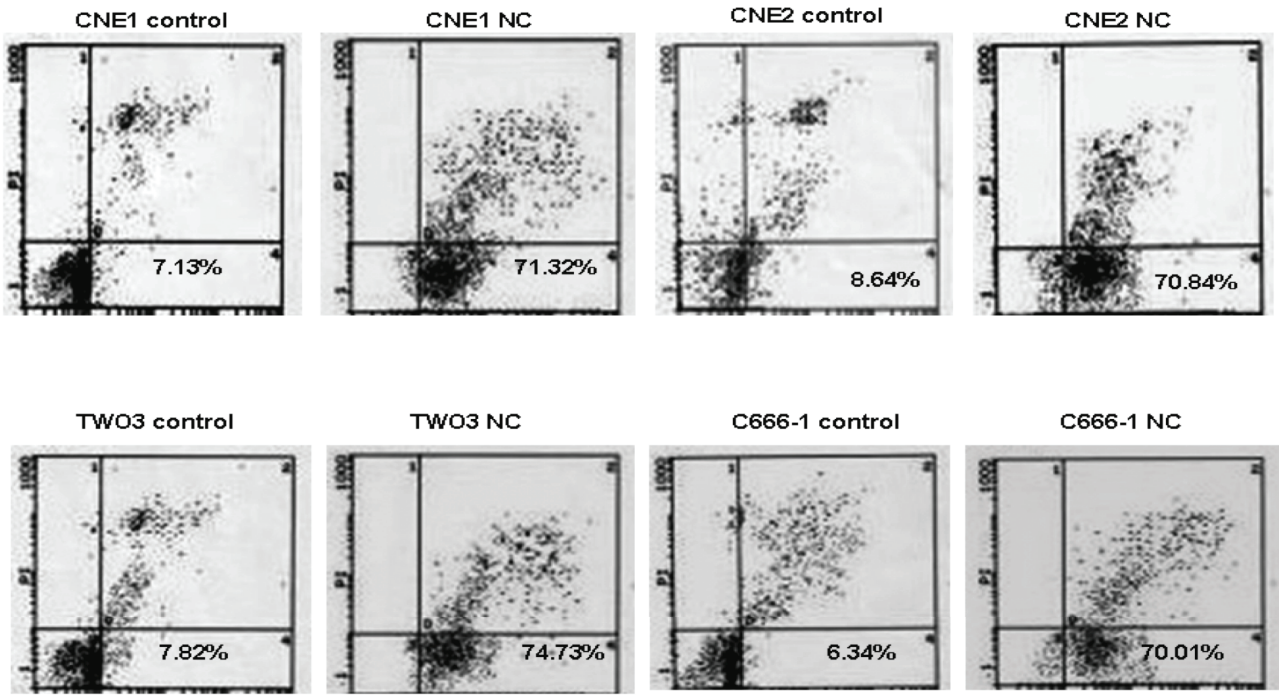

Figure 2. Flow cytometric detection of nitidine chloride of apoptosis of 4 types of nasopharyngeal carcinoma cell lines. Flow cytometry was used to measure cell apoptosis. Four cell lines (CNE1, CNE2, TWO3, and C666-1) were treated with nitidine chloride at different concentrations for 12 h. The apoptotic rates were detected and compared with the control group. The apoptosis rates of CNE1, CNE2, TWO3 and C666-1 cells treated with nitidine chloride were 71.32, 70.84, 74.73 and 70.01%, respectively, significantly higher compared with the control groups (p<0.01). Thus, nitidine chloride induced apoptosis in nasopharyngeal carcinoma cells.

fect on p53 mRNA and protein expression of nasopharyngeal carcinoma cells. As shown in Figure 3A, semi quantitative RT-PCR was applied for the determination of p53 mRNA expression of CNE1 cells. After 24 h treatment with 10, 20, 30 and 40 mg/l NC, p53 mRNA increased as the drug concentration increased compared with the control group. By gel imaging analysis and analysis of gray scale scanning, the ratio was statistically significant (p<0.01 and p<0.05, respectively). Western blot was applied for determination of p53 protein expression. As shown in Figure 3B, after 24 h treatment of 10, 20, 30 and 40 mg/l, p53 protein expression increased with increasing drug concentration compared with the control group. Consistent with the above RT-PCR results, gel imaging analysis system and gray scan analysis showed a statistically significant ratio (p<0.01 and p<0.05, respectively).

Discussion

Animal and plant derived natural compounds are becoming more and more popular for the study of anticancer drugs [11-13]. Antitumor effects of traditional Chinese Medicine have been shown for trichosanthin (TCS) in nasopharyngeal carcinoma [14], gambogic acid (GA) in hepatocellular carcinoma [15] and ursolic acid (UA) in colorectal cancer [16]. Because plant extracts have become an important source for new antitumor drugs, the South America Antitumor Drug R & D office had set certain related standards, and an *in vitro* plant extract with $IC_{50} \leq 50$ mg·L^{-1} is identified as effective antitumor component [17]. The roots of *Zanthoxylum nitidium* (Roxb.) DC., one of the traditional Chinese medicines, have long been used for the treatment of rheumatic arthritis, periodontitis,

and empyrosis, as well as for their general analgesic properties. NC, which was used in the present study, is a pentacyclic alkaloid isolated from *Z.nitidium*. It has been identified as a potential antitumor drug because of its topoisomerase I/II inhibiting activity in some cancers [4,18,19]. Apart from its identified immune regulation function [20], it possesses antioxidant properties, reduces tissue damage and promotes tissue repair [7,21]. Though its specific pharmacological mechanism is unclear, it has been reported that it interferes with the regulation of cell immune function, oxidation resistance and damage repair, and induces tumor cell apoptosis [4,22]. In the present study, we found that NC could significantly suppress the tumor cell growth, by inhibiting proliferation and inducing apoptosis of the nasopharyngeal carcinoma cells.

Apoptosis is a kind of cell death through a series of death signaling molecules [23-25]. It has been shown that many antitumor drugs function by inducing tumor cell apoptosis rather than necrosis. Induction of tumor cell apoptosis is of great importance in cancer therapy and is the antitumor cell inhibition mechanism of many chemotherapeutic drugs. Thus, the efficacy of chemotherapy may be improved by developing drugs that can induce apoptosis of tumor cells [26-28]. In the present study, inverted microscopy showed typical apoptosis of cancer cells, suggesting the apoptotic effect of NC on nasopharyngeal carcinoma cell lines CNE1, CNE2, TWO3 and C666-1. Further detection of flow cytometer showed clear apoptosis of nasopharyngeal carcinoma cell lines after NC treatment with statistical significance when compared with the control group ($p<0.01$). In addition, MTT assay used in this study showed the proliferation inhibition effect of NC on CNE1, CNE2, TWO3 and C666-1 cell lines with dose- and time-dependent relationship.

To further study the proliferation inhibition and apoptosis induction of NC on nasopharyngeal carcinoma cells, CNE1 cell line was selected as representative for the examination of the pro-apoptotic gene p53 mRNA and protein expression levels. After drug treatment, mRNA and protein expression levels of p53 were found significantly increased, suggesting the growth and/or apopto-

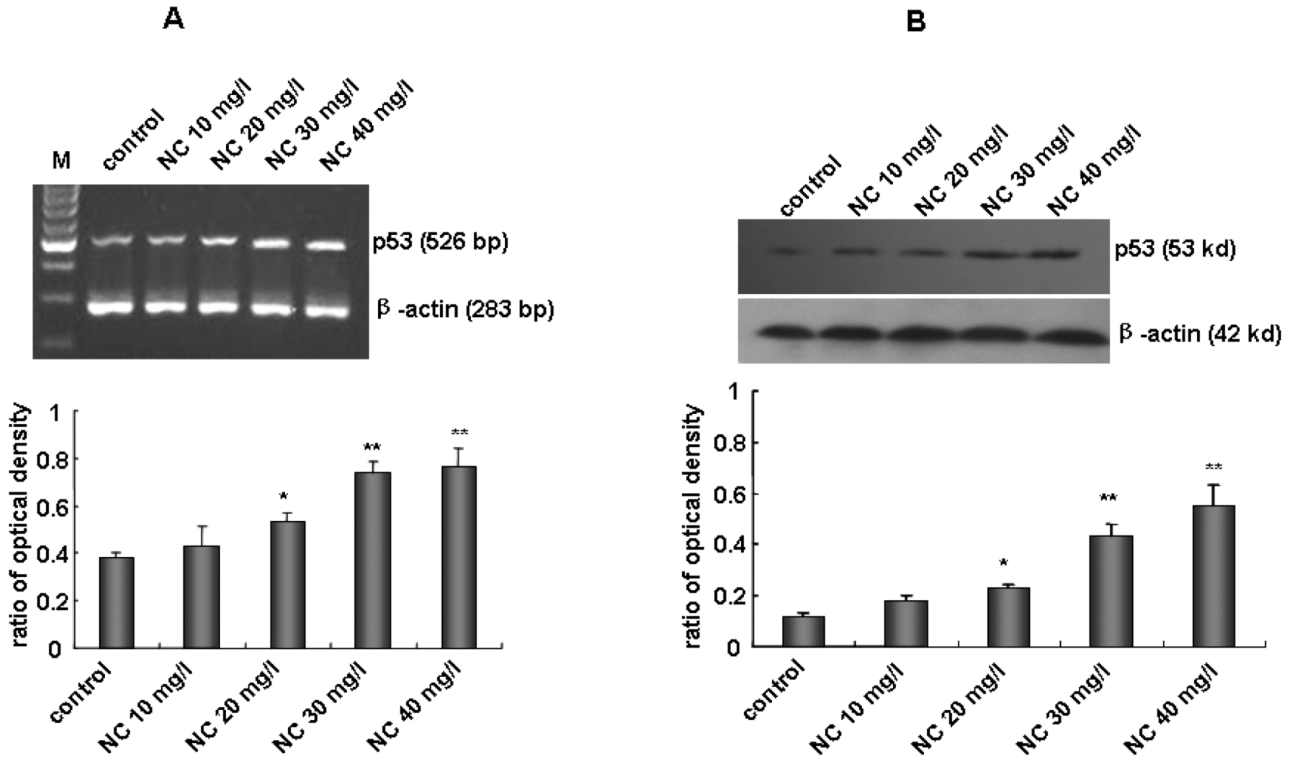

Figure 3. The effect of nitidine chloride on p53 expression in CNE1. The CNE1 cell line was treated with different concentration of nitidine chloride (10, 20, 30 and 40 mg/l) for 24 h. The p53 mRNA and protein expression in nasopharyngeal carcinoma cells were detected by RT-PCR and Western blotting, respectively. **A:** The effect of nitidine chloride on p53 mRNA of nasopharyngeal carcinoma cell line CNE1. *$p<0.05$ vs normal; **$p<0.01$ vs normal. M: DNA marker. **B:** The effect of nitidine chloride on p53 protein expression of nasopharyngeal carcinoma cell line CNE1. *$p<0.05$ vs normal; **$p<0.01$ vs normal. The results showed that the mRNA and protein expression levels of p53 increased significantly after nitidine chloride treatment.

sis inhibition of NC on nasopharyngeal carcinoma cells was closely associated with increased expression of p53. In other words, p53 at least partially mediated the proliferation inhibition and induction of apoptosis of NC on nasopharyngeal carcinoma cells. The question whether other molecules had participated in this regulation needs further exploration.

Acknowledgement

This study was supported by grants from the Department of Guangxi Science and Technology, China. (No.0322024-1A), the National Natural Science Foundation of China (No.30871186), the Health Department Funded Self Financing Project of Guangxi Zhuang Autonomous Region (Z2012066) and the Youth Foundation of Guangxi Medical University (No.02604001020).

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